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PURIFICATION AND CHARACTERIZATION OF EQUINE
PLATELET TROPOMYOSIN

by

GRAHAM PETER COTE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
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EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Purification and Characterization of Equine Platelet Tropomyosin" submitted by Graham Peter Cote in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

ABSTRACT

Two new methods for the isolation of large amounts of a non-muscle tropomyosin (Tm) from horse blood platelets have been developed. One method, which does not subject the protein to denaturing conditions, involves successive homogenization of the platelets in a Waring Blender with 0.1M NaCl and 1.0M NaCl buffered with 10 mM Tris, 2.5 mM EDTA, pH 8.0. Following several isoelectric precipitations (pH 4.6) the platelet Tm was purified to greater than 95% homogeneity (as judged by SDS gels) with a 20% yield by DEAE cellulose and hydroxyapatite chromatography. The second method involves an extraction of the platelets with 0.1M NaCl, 2.5 mM EDTA, 25 mM HCl, pH 2.0, followed by an isoelectric precipitation at pH 4.4. The pH 4.4 precipitate is extracted with 0.1M NaCl, 10 mM Tris, 2 mM DTT, 2.5 mM EDTA, pH 7.8, and the platelet Tm further purified by isoelectric precipitation, a boiling step and hydroxyapatite chromatography to greater than 95% homogeneity with a 30% yield. SDS gel electrophoresis indicated that the platelet protein, like other non-muscle Tms, had a lower subunit molecular weight than muscle Tm (28,500 compared to 33,000 daltons). Evidence for two different forms of the platelet Tm was obtained by SDS urea gel electrophoresis. Circular dichroism, ultracentrifugation, gel filtration and paracrystal studies all indicated that the platelet protein, like muscle Tm, was an asymmetric α -helical coiled-coil. The complete amino acid sequence of the platelet Tm has recently been completed (Lewis, W.G., Cote, G.P. and Smillie, L.B.), and confirms that platelet Tm retains all the structural features of skeletal Tm which lead to the formation of a stable coiled-coil structure. Unlike muscle Tm, the platelet

appears to be unable to form head-to-tail aggregates at low ionic strength as estimated from viscosity measurements. This lack of self association can probably be attributed to the markedly different amino and carboxyl terminal sequences of the platelet Tm when compared to skeletal Tm. Platelet Tm has also lost the ability to bind to muscle Tn-T as shown by affinity chromatography and viscosity measurements, even though the amino acid sequence in the region of Cys 190, the position where Tn-T is thought to bind to skeletal Tm, has not been significantly changed in platelet Tm. This finding suggests that Tn-T binds closer to the carboxyl terminus of skeletal Tm than previously thought, perhaps in the region of residues 257-284, one of the areas most altered in platelet Tm. Platelet Tm, probably as a result of its shorter length (by 38 residues), does not bind to skeletal muscle actin or platelet actin with as high an affinity as skeletal Tm. Binding experiments, performed by the cosedimentation method, demonstrate that under physiological conditions (150 mM KCl, 1 mM Mg⁺⁺) platelet Tm binds very poorly to F-actin. Binding to F-actin could be induced by the addition of Mg⁺⁺ (to 6 mM), by myosin S-1 heads (in the absence of ATP), or by the addition of Tn-I. The ability of platelet Tm to replace skeletal Tm in a skeletal muscle myosin S-1:actin ATPase system was tested. Under the assays conditions used (30 mM KCl, 5 mM MgCl₂, 2 mM DTT, 2 mM ATP, 0.1 mM EGTA, 2 mM Tris, pH 7.8) platelet Tm did not bind to actin and had no effect on the S-1 ATPase. However, addition of troponin induced the binding of platelet Tm to actin and good inhibition in the absence of Ca⁺⁺ was obtained. This inhibition could be only partially released (40 to 50%) upon addition of Ca⁺⁺

probably because of the missing connection between platelet Tm and Tn-I. Good inhibition of the actin activated ATPase of S-1 could be obtained by replacing whole troponin with Tn-I alone, suggesting that platelet Tm still retains the ability to interact with this subunit of troponin. The inhibition observed with platelet Tm and Tn-I was released upon addition of Tn-C whether or not Ca^{++} was present. This release of inhibition correlated with binding studies which indicated that Tn-C could reverse the ability of Tn-I to induce the binding of platelet Tm to actin, with or without Ca^{++} . If calmodulin (purified from bovine brain) was added in place of Tn-C good Ca^{++} sensitive inhibition of the S-1 ATPase could be obtained with platelet Tm and Tn-I. In the absence of Ca^{++} calmodulin allowed Tn-I to induce the binding of platelet Tm to actin, but once Ca^{++} was added platelet Tm was removed from the actin filament. These results suggest that non-muscle Tm, an inhibitory protein, and calmodulin may function as a Ca^{++} sensitive regulatory system for contractile events in non-muscle cells by means of an on-off mechanism, where "on" corresponds to the inhibited state with the Tm and inhibitory protein bound to actin, and "off", the activated state, is equivalent to free F-actin filaments.

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LIST OF ABBREVIATIONS

A_λ	absorbance of a protein at the given wavelength λ
ABP	actin binding protein
ATP	adenosine triphosphate
CB-1	a fragment of Tn-T, residues 1 to 151
CD	circular dichroism
DEAE	diethylaminoethyl
DTT	dithiothreitol
$E_\lambda^{1\%}$	absorbance of a 1% protein solution in a 1 cm path-length cell at the wavelength λ
EDTA	Ethylenediamine-tetraacetic acid
EGTA	ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetraacetic acid
f_H	fractional value of α -helix in a protein
g	grams
x g	times gravity
HMM	heavy meromyosin
K_d	fraction of the gel internal volume accessible to a protein
Mg^{++}	ATPase the ATPase of myosin which is activated by actin
M.Wt.	molecular weight
PMSF	phenylmethanesulfonyl fluoride
S-1	soluble single headed fragment of myosin
S-2	coiled-coil hinge region of myosin
SDS	sodium dodecyl sulfate
TA	fragment of platelet Tm corresponding to residues 183-238
Tm	tropomyosin
Tn-C	the calcium binding subunit of troponin

Tn-I	the inhibitory subunit of troponin
Tn-I-C	the complex of Tn-I with Tn-C
Tn-I-Cal	the complex of Tn-I with calmodulin
Tn-T	the Tm binding subunit of troponin
TPCK	Tosylphenylalanine chloromethyl ketone
Tris	tris-(hydroxymethyl) aminomethane
UV	ultraviolet
\bar{v}	the partial specific volume
ϵ_m	the molar extinction coefficient
$\Delta\epsilon$	the difference in the molar extinction coefficients for left and right circularly polarized light
λ	wavelength
$[\Theta]_\lambda$	mean residue ellipticity at the wavelength λ
η_{rel}	the relative viscosity
ρ	the solvent density

CHAPTER I

INTRODUCTION

One basic system, composed of two proteins, actin and myosin, has evolved in nature to perform the fundamental process of converting chemical energy into motion. This system is present in virtually all organisms, ranging from the protozoa and higher plants to the mammal and, perhaps, even to the prokaryotes (Nakamura & Watanabe, 1978). Within the more complex organisms, such as the mammal, every type of cell, regardless of its specialized function, contains actin and myosin. The knowledge that we have of the structure and function of actin and myosin, and of the proteins associated with them, come almost entirely, however, from studies on a single tissue: skeletal muscle.

Skeletal muscle is a highly specialized tissue designed to produce movement and force in a specific direction, and for this purpose it contains large amounts of actin and myosin organized into closely packed, highly ordered arrays (Fig. 1). This organization has greatly facilitated studies on the mechanism of action of actin and myosin on a biochemical and molecular level. Fortunately, it appears that much which is known of the skeletal muscle system is directly relevant to the more complex contractile systems of the thousand of types of non-muscle cells.

A. SKELETAL MUSCLE

There have been many good reviews published on the proteins

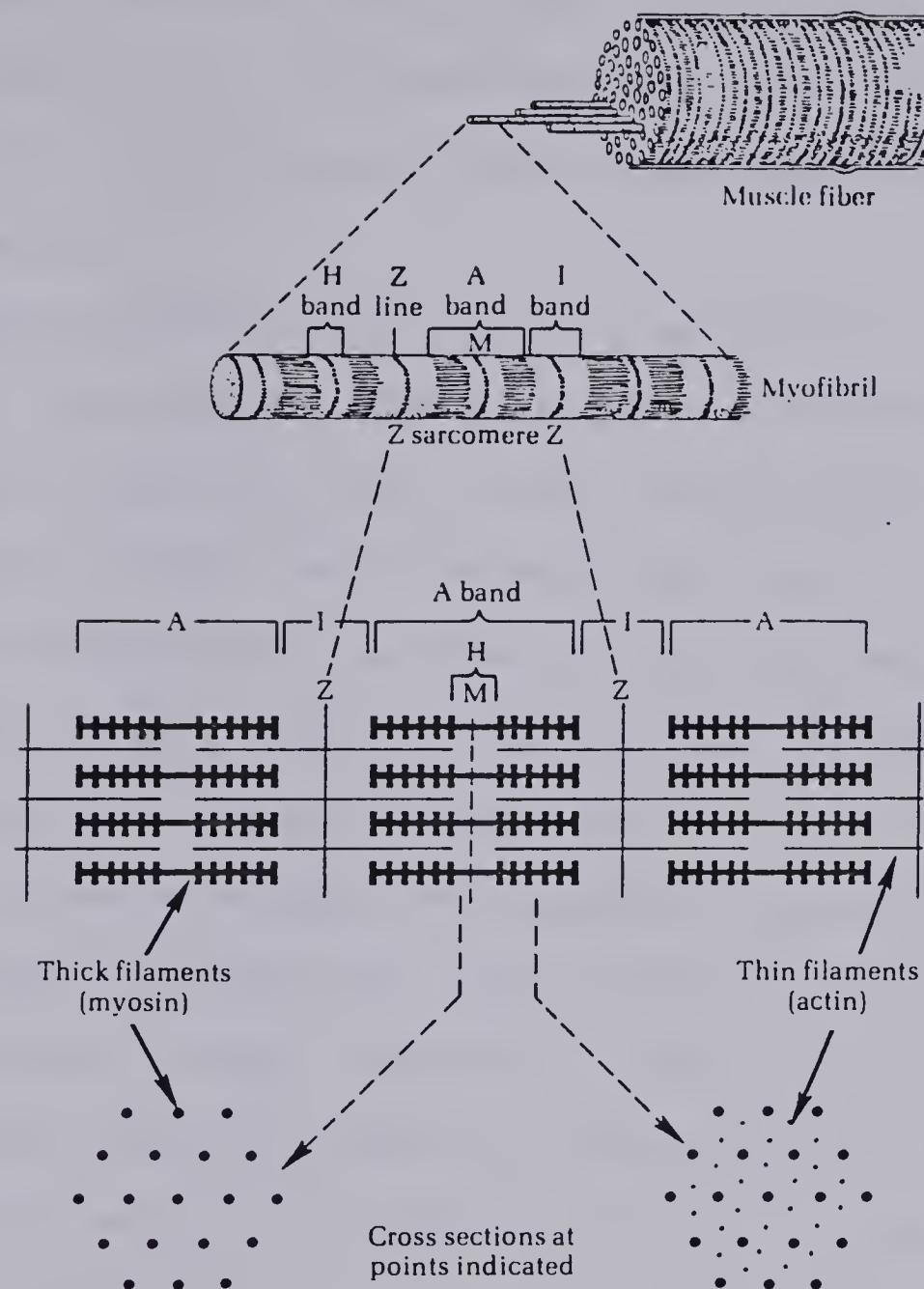


Fig. 1. Schematic diagram of the structure of skeletal muscle.
[from Lehninger, 1975]

of skeletal muscle, including those by Taylor (1979), Mannherz and Goody (1976), Squire (1975), and Weber and Murray (1973). These can be referred to for more information on many of the topics discussed in this introduction.

In mammalian skeletal muscle, myosin is aggregated to form the thick filaments, while the thin filaments, which are attached at one end to structures called the Z lines, contain actin as well as the regulatory proteins troponin and tropomyosin (T_m) (Fig. 2). When muscle contracts the thick and thin filaments slide past each other, the actin being drawn further into the array of myosin filaments. The end result is that although the lengths of the filaments remain constant, the Z lines are brought closer together, shortening the muscle. This sliding filament model, first proposed by Huxley and Hanson on the basis of electron microscopic studies, is now generally accepted as being the means by which actin and myosin produce movement, or force, not only in muscle, but in all types of cells.

1. Actin and Myosin

How such movement is produced requires detailed knowledge of the structures of actin and myosin, and of the way in which these two proteins interact.

Purified actin is a globular protein of 42,000 M.Wt., and is found as a monomer, G-actin, only in the absence of divalent cations at low ionic strength (reviewed by Oosawa & Kasai, 1971). G-actin tightly binds one molecule of nucleotide (usually ATP) and one divalent cation (probably Mg^{++} in vivo), both of which are essential for the stabilization of the protein's tertiary structure.

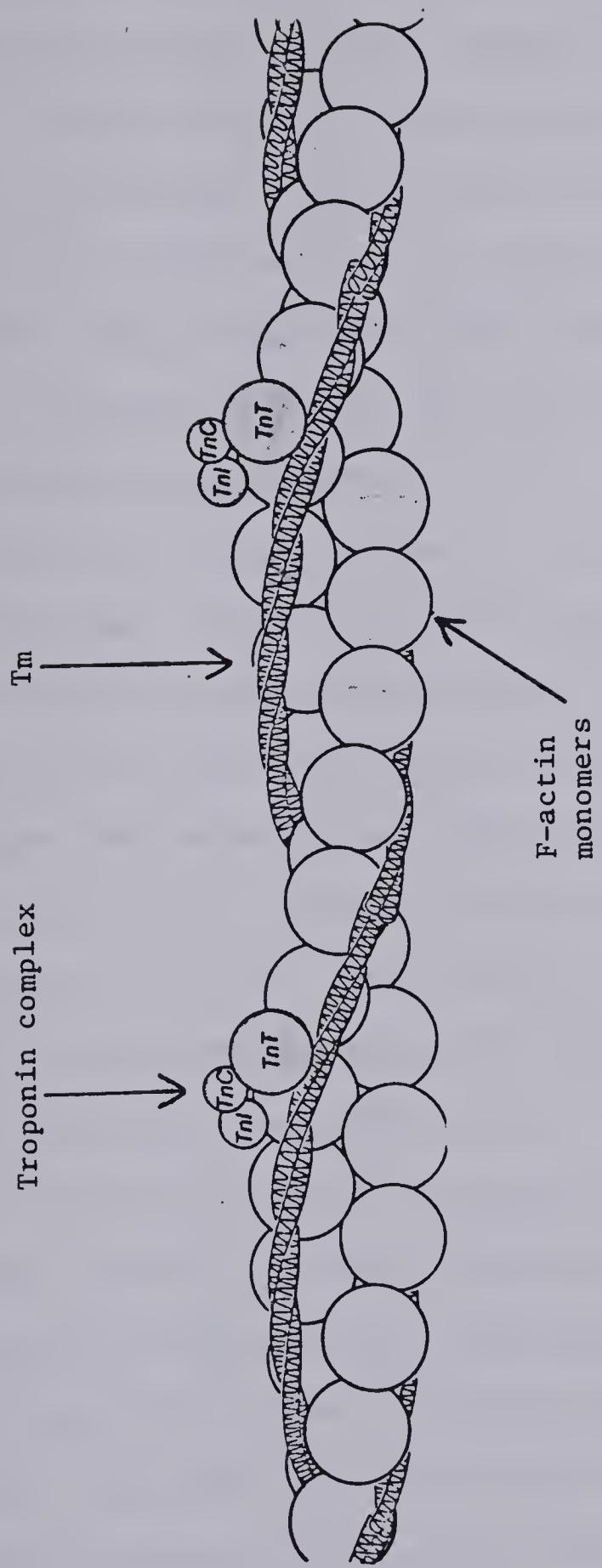


Fig. 2. Model for the structural organization of the thin filament proteins of skeletal muscle. There is one molecule of troponin and one of Tm for every seven F-actin monomers. [From Cohen, 1975].

G-actin, if present in amounts exceeding a certain threshold concentration, termed the critical concentration, will spontaneously polymerize to F-actin, a double stranded, helical filament several microns in length (Fig. 3). The critical concentration decreases rapidly as the amount of salt present increases (Fig. 4). Under physiological conditions actin is present almost entirely as F-actin filaments. This is the form in which actin is found in the thin filaments of muscle, and the only form of actin which is capable of participating in contractile events.

Each actin monomer in the thin filament is capable of binding one myosin head. Myosin is a large protein consisting of two heavy chains (200,000 daltons each) and four light chains. The carboxyl terminal parts of the myosin heavy chains are highly α -helical and wind around each other to form a coiled-coil, while the amino terminal regions are folded to produce a globular domain, or head, of about 90,000 daltons (Fig. 5). Each head has bound to it one DTNB light chain of 18,000 M.Wt. and one alkali light chain with a M.Wt. of either 21,000 (A-1), or 16,500 (A-2).

The globular head is the portion of myosin which binds actin and which contains the ATPase activity of the molecule. The alkali light chains are essential for the activity of myosin while the DTNB light chains can be removed without any apparent effect on its ATPase activity. The myosin heads can be split from the rod region by proteolytic enzymes to yield either a two-headed heavy meromyosin (HMM), or two single-headed S-1 fragments, all of which retain the full enzymatic activity of myosin (Lowey et al., 1969).

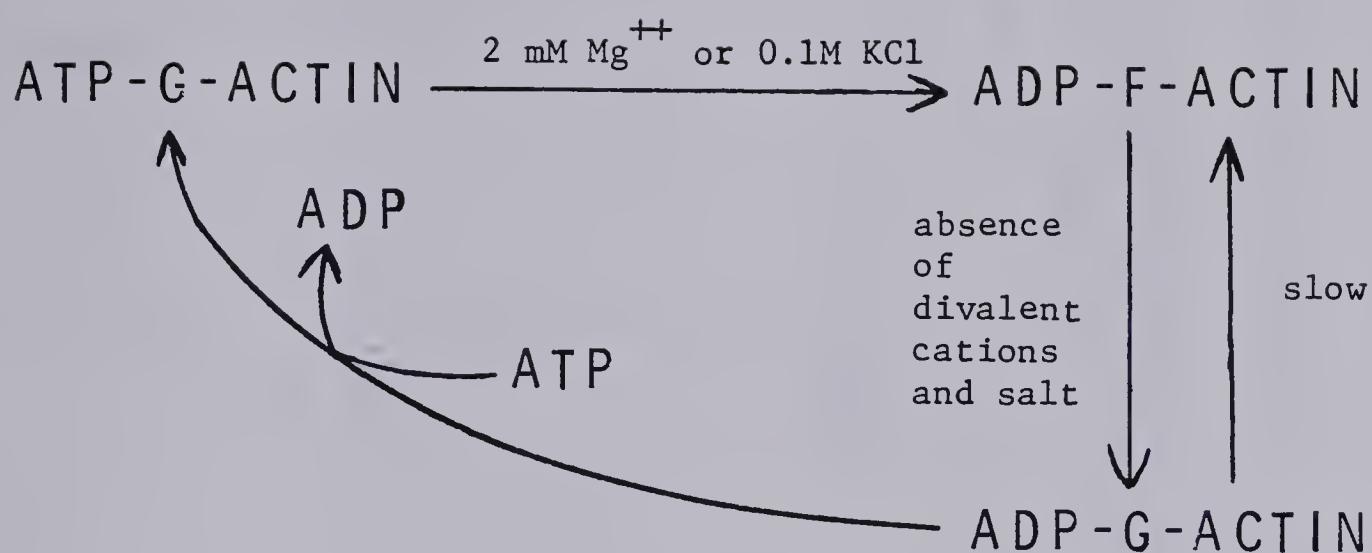


Fig. 3. The polymerization and depolymerization of actin. Monomeric G-actin spontaneously polymerizes to F-actin (the form of actin shown in Fig. 2) upon addition of salt or divalent cations. The bound ATP is hydrolyzed to ADP as G-actin polymerizes. This reaction is not absolutely necessary for G to F transformation, but does appear to accelerate the process. The cycle illustrated can be repeated any number of times simply by changing the solution conditions.

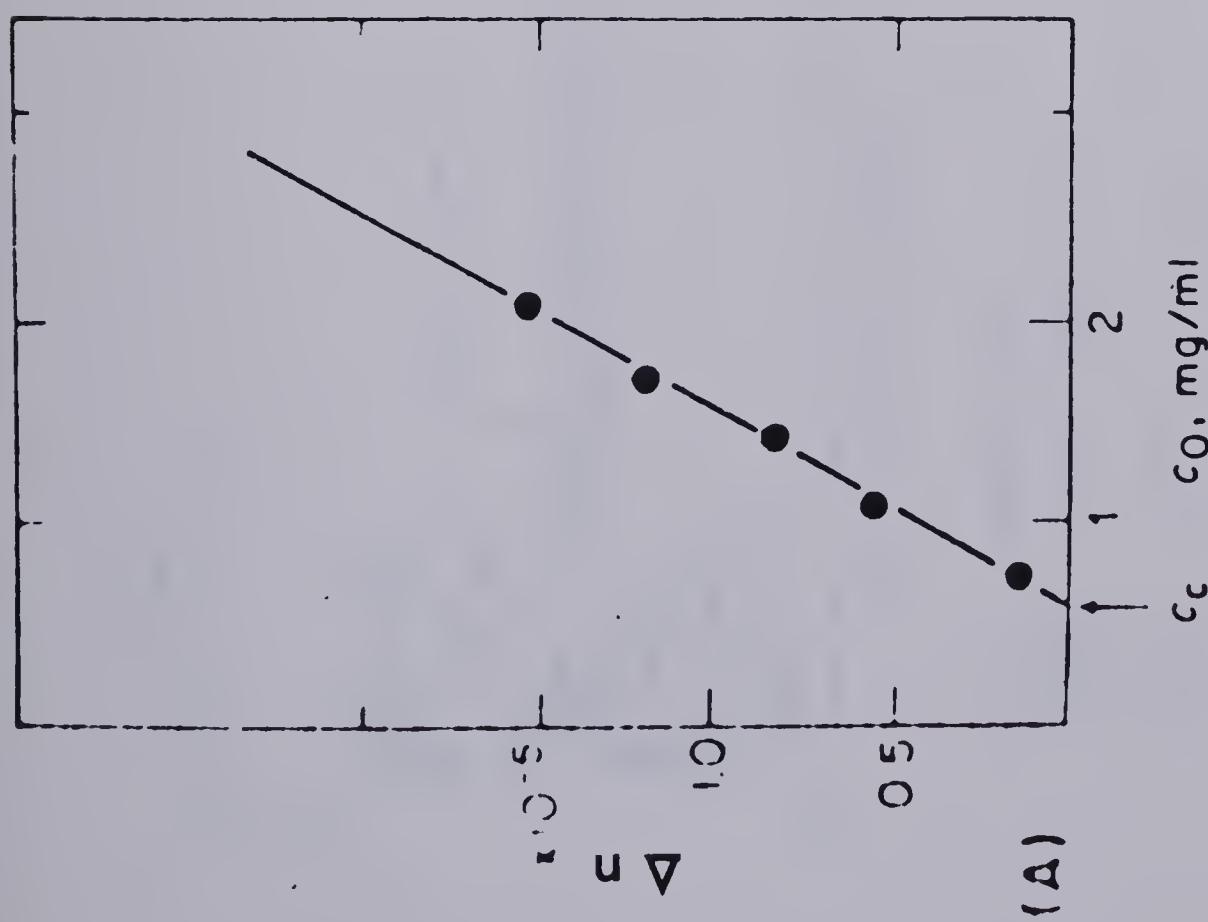


Fig. 4a. The degree of flow birefringence (Δn), which is proportional to the amount of F-actin present, as a function of the total actin concentration (c_0). Below a critical actin concentration (c_c), no F-actin is formed, while above this concentration all G-actin will be incorporated into actin filaments. The buffer was 0.8 mM MgCl₂, 8 mM Veronal-HCl, pH 8.7.

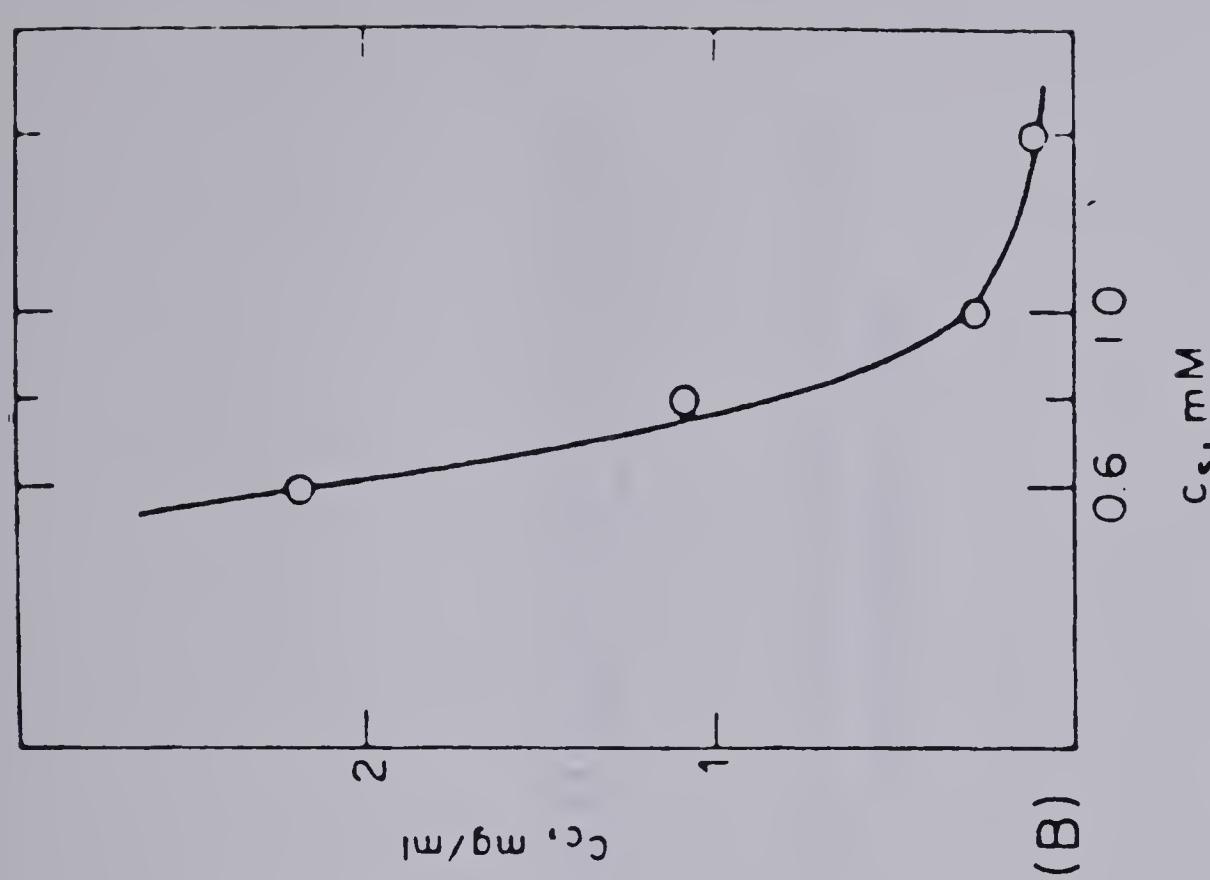


Fig. 4b. The critical actin concentration as a function of the amount of MgCl₂ present (c_s). In 1 mM MgCl₂ the critical concentration is below 1 μ M.
Both figures from Oosawa and Kasai (1971)

S-1 (Head)

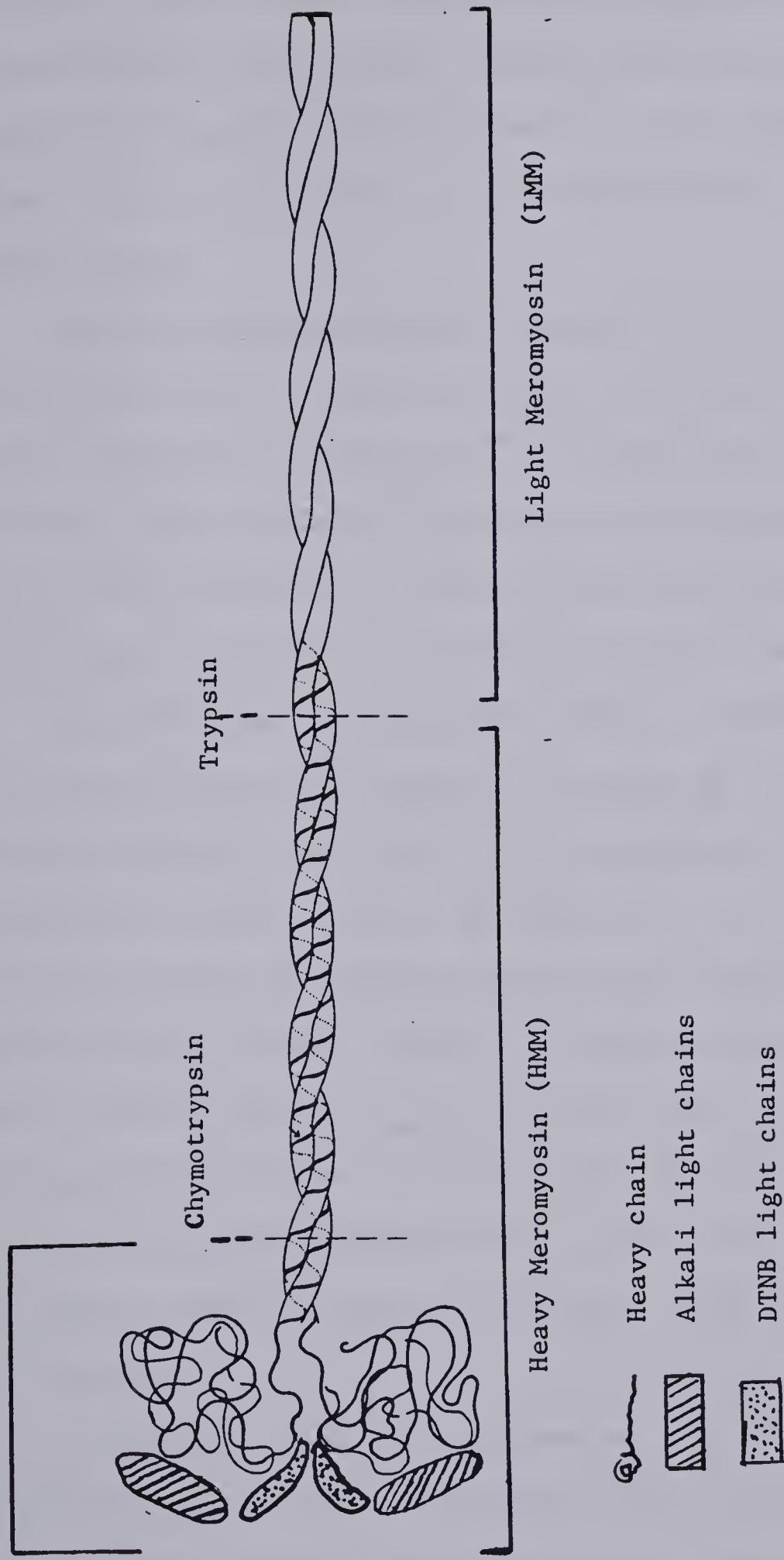


Fig. 5. Diagrammatic representation of the myosin molecule. The molecule is composed of six polypeptide chains; two heavy chains and two pairs of light chains. S-1, HMM, and LMM are fragments generated by limited proteolysis at the points indicated.

These globular fragments are often used in studying the function of myosin, since they have the advantage of being soluble at physiological ionic strength. Whole myosin, under such conditions, aggregates through the coiled-coil region to form large bi-polar filaments that are equivalent to the thick filaments observed in skeletal muscle.

Myosin is an enzyme which hydrolyzes ATP to ADP + PO₄, but alone, without actin, it is only active in solutions of high ionic strength (0.5M KCl) with either Ca⁺⁺ or EDTA present. These activities are greatly inhibited by Mg⁺⁺ and have no physiological significance, although they are useful in following the purification of myosin or in comparing the activities of different myosin preparations.

The ATPase activity of myosin that is involved in contractile events occurs at low ionic strength, requires Mg⁺⁺, and is exclusively activated by actin (Fig. 6). At very high actin concentrations the myosin Mg⁺⁺ ATPase rate can be activated by up to 200 times, and a level of activity is reached similar to that found in working muscle (Eisenberg & Moos, 1968). Increasing the ionic strength lowers the apparent binding constant, K_{app}, of myosin heads to actin, thereby lowering the ATPase rates for finite actin concentrations (Fig. 6) (Eisenberg & Moos, 1968; Wagner et al., 1979). For this reason in vitro ATPase assays are usually performed at lower than physiological ionic strength.

The contraction of skeletal muscle is thought to be produced by a cyclic process in which the myosin heads, projecting from the thick filaments, attach to the thin filaments. Once attached, the

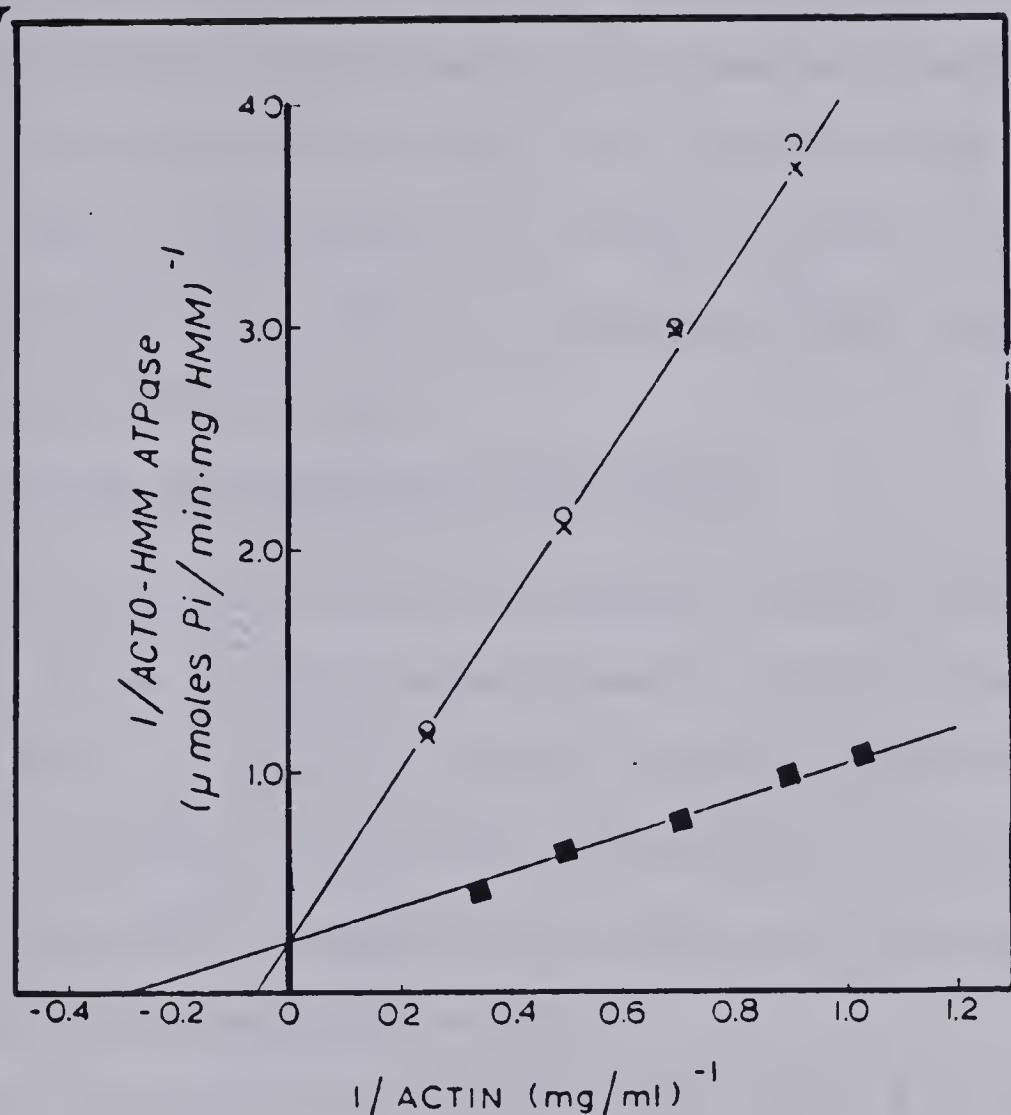


Fig. 6. The reciprocal of the actin activated ATPase of HMM is plotted against the reciprocal of the added actin concentration. Samples marked (x,o) contained 80 mM KCl, while those designated (■) contained 40 mM KCl. V_{max} remains constant, but K_m decreases at the higher ionic strength. Samples marked (x) contained 2 mM ATP, 1 mM $MgCl_2$, while those marked (o) contained 0.5 mM ATP, 0.25 mM $MgCl_2$, both at the same ionic strength. The fourfold change in ATP concentration had no effect on the ATPase activity.
[from Eisenberg & Moos, 1968]

heads, with energy gained from the hydrolysis of ATP, rotate relative to the actin filament so that a sliding motion is produced (Huxley, 1969, 1976). An alternative force generating event, proposed by Harrington (1972), would be an α -helix to random coil transition within the coiled-coil S-2 region of myosin, brought about by the hydrolysis of ATP on a neighbouring head. Both of these models are shown in Fig. 7.

2. Regulation of the Actin-Myosin Interaction

The interaction of actin with myosin is controlled, in skeletal muscle, by two proteins: troponin and Tm. These proteins, attached to the thin filament, respond to changes in the concentration of free Ca^{++} to either block or allow contraction. At levels of free Ca^{++} below about 10^{-6} M, troponin and Tm prevent the binding of myosin heads to actin, and therefore contraction.

Tm is a rod-like protein, 42 nm long, with a M.Wt. of 66,000, composed of two highly (>90%) α -helical subunits wrapped around each other to form a coiled-coil (reviewed by Smillie, 1979). Two homologous forms of the Tm subunit, termed α and β , which differ slightly in amino acid sequence, have been found in skeletal muscle (Cummins & Perry, 1973; Mak et al., 1979). The functional differences between the two chain types have not yet been determined, but it is known that the β form is present in larger amounts in embryonic, and slow, skeletal muscle (Cummins & Perry, 1973; Roy et al., 1978).

Each Tm molecule binds to seven actin monomers on the thin filament, and, as well, interacts with one molecule of the troponin complex. This latter protein is composed of three subunits, one of

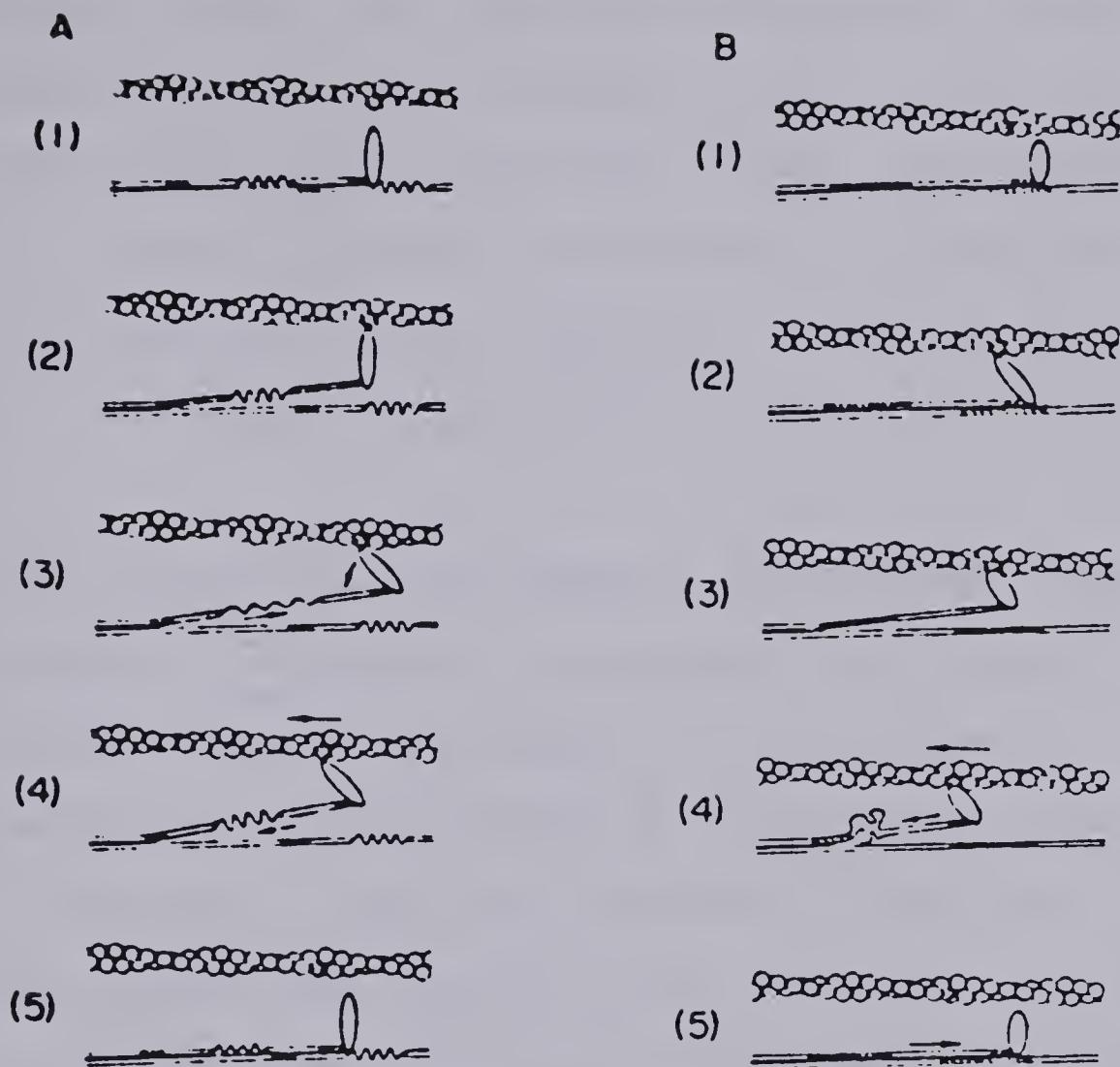


Fig. 7. Two models by which the interaction of myosin with actin may produce contraction. (A) Huxley-Simmons model: 1, resting state; 2, attachment of S-1 to actin filament; 3, rotation of S-1 while it is attached to the actin filament and simultaneous stretching of the spring-like elastic component in S-2; 4, power stroke resulting from retraction of elastic component; 5, return of cross-bridge to resting state. (B) Helix-coil model: 1, resting state; 2, S-1 swivels to attach to actin filament; 3, release of S-2 from thick filament surface; 4, power stroke resulting from helix-coil transition in S-2; 5, return of cross-bridge to resting state.
[from Tsong et al., 1979]

which, Tn-T (M.Wt. 31,000), binds specifically to Tm. The other two components (Tn-I and Tn-C) each perform an essential function in the regulation of contraction (reviewed by Perry et al., 1979; Ebashi, 1974; Gergely, 1976). Tn-I (M.Wt. 21,000), in co-operation with Tm, is capable of inhibiting the interaction of actin with myosin, while Tn-C (M.Wt. 18,000) confers Ca^{++} sensitivity to the inhibition by its ability to specifically interact with Tn-I and Ca^{++} .

Upon binding Ca^{++} , Tn-C undergoes a conformational change, which is transmitted through the Tn-T component to Tm. The end result is that Tm changes its position on the actin filament, rolling deeper into the groove between the two strands of actin. Somehow, this movement releases the inhibition of contraction, and myosin heads are once again able to interact with actin. The movement of Tm over the actin surface may physically unblock the binding site for myosin (which, in the absence of Ca^{++} , would have been covered), or may act by inducing conformational changes in the actin monomers (Eaton, 1976). In either case, Tm is able to amplify the effects of Ca^{++} binding, so that one molecule of troponin is capable of regulating seven actin monomers at one time.

A more detailed discussion of the structures and biological activities of Tm and troponin will be given, at the appropriate points, later in the thesis. However, with this brief introduction to the basic features of the contractile proteins of muscle, we can now turn to an examination of the non-muscle systems.

B. NON-MUSCLE CONTRACTILE PROTEINS

Most of our present knowledge concerning the properties and functions of contractile proteins in cells other than muscle has been gained within the last decade. The rapid progress and great upsurge of interest in this field has lead to the publication of a large number of reviews and books covering all aspects of the subject.

General reviews on non-muscle contractile proteins include those by Korn (1978), Stossel (1978), Clarke and Spudich (1977), Hitchcock (1977), and Pollard and Weihing (1974), while collections of reviews or the proceedings of symposia dedicated to this topic can be found in the books Methods and Achievements in Experimental Pathology, vols. 8 and 9 (Ed. Gabbiani, 1979); Cell Motility, Books A and B, Cold Spring Harbor Conference on Cell Proliferation, vol. 3 (Eds. Goldman et al., 1976); Contractile Systems in Non-Muscle Tissues (Eds. Perry et al., 1976); and Molecules and Cell Movement (Eds. Inoue & Stephens, 1975). The reader is referred to these references for a more detailed discussion of much of the information presented in this part of the introduction.

Since platelet Tm, in analogy to muscle Tm, presumably performs its biological role of regulating the interaction of actin and myosin only when attached to F-actin, the emphasis in the following review will be on what is known of the non-muscle actins, and of the proteins associated with them. As well, the various means by which contraction may be regulated in non-muscle cells will be considered.

Since this thesis is concerned with the isolation and characterization of a non-muscle Tm from platelets, it would, at first, seem desirable to limit the scope of this section of the introduction to include only a discussion of the contractile proteins of platelets. However, if such a limitation is imposed, it would mean leaving out much important, and possibly relevant, information, because at present research into the nature of non-muscle cell motility is not focussed onto any one particular organism, but rather is spread out over a wide variety of non-muscle systems, ranging from the amoeba and the slime mold all the way to a large number of mammalian tissues. By discussing only the contractile proteins of platelets all the valuable studies performed on other systems (many of which have not been repeated using platelets) would be neglected, and the result would be a very incomplete picture of non-muscle motility.

Fortunately, it would appear, from our present state of knowledge, that the contractile proteins of many different non-muscle cell types bear striking resemblances to each other. Not only actin and myosin, but many other proteins involved in contraction seem to have been highly conserved. For example, profilin, a small protein which prevents the polymerization of G-actin, has been identified in mammalian spleen, brain, thymus, lymphocyte and platelet cells, as well as in the amoeba Acanthamoeba. Similar patterns of widespread distribution appear to be the rule rather than the exception for proteins involved in motility. In particular, preliminary evidences indicates that most mammalian non-muscle Tms may be very similar (Fine & Blitz, 1975).

It would seem reasonable then to take the position that platelet Tm is a typical representative of any non-muscle Tm, and, as well, to assume that contractile proteins or control mechanisms present in other non-muscle cells are probably also active in platelets. It is realized that this position is an oversimplification, for there must be differences in the processes which organize and arrange the contractile proteins in each type of cell, but these are not yet known.

For reviews which specifically cover the contractile proteins of platelets and the manner in which they act, see Cohen (1979), White and Gerrard (1979), and articles in the book *Platelets in Biology and Pathology* (Ed. Gordon, 1976).

1. Functions of the Contractile Proteins

The contractile proteins are present in quite large amounts in many non-muscle cells (Table I) and are responsible for many processes involving movement. These include cell motility (ex. amoebae, slime molds, leucocytes), phagocytosis (ex. macrophages, amoebae), secretion by exocytosis (ex. nerve, pancreas, adrenal cells), and force production (ex. platelets and fibroblasts at the site of a wound).

Besides such specialized functions, the contractile proteins are involved in essential processes common to the large majority of eukaryotic cells, including the pulling apart of chromosomes, the division of the cell into two during mitosis (Schroeder, 1975), and the movement of materials and organelles from one part of the cell to the other. The latter example can be best illustrated by

TABLE I

Actin and Myosin Contents of Various Cells
(% of Total Protein)

Cell Type	Actin	Myosin	Reference
Cultured Fibroblast	8 - 9	2 - 3	Bray and Thomas (1975)
Chick Embryo Brains	6	-	Ostlund et al. (1974)
Macrophages	10' - 12	2	Stossel and Hartwig (1976b)
<u>Physarum</u>	2 - 5	0.5 - 1	Jacobsen et al. (1976)
Human Platelet	10	1	Stossel (1978)
<u>Dictyostelium</u>	7	0.5	Stossel (1978)
Smooth Muscle	25	8	Stossel (1978)
Skeletal Muscle	23	38	Stossel (1978)

the cytoplasmic streaming in the green algae Nitella (Palevitz, 1976), or by the axoplasmic transport of products from the cell body to distant synapses in neurons (Durham, 1974).

Actin filaments (microfilaments) are often found in close contact with the plasma membrane of a cell (probably attached to it through an as yet unidentified protein), and are used in the construction of ruffles, pseudopods and other membrane structures. In many cells a gel-like region, called the cortex, from which organelles are excluded, occurs just beneath the plasma membrane, and is formed from a meshwork of microfilaments (Schroeder, 1975), which may, either through direct or indirect connections, affect the anchorage and mobility of proteins on the cell surface (Edelman, 1976; Sundquist & Ehrnst, 1976). For example, it appears that actin and myosin are involved in the capping process, whereby cell surface proteins which have been crosslinked by divalent antibodies or lectins are systematically gathered within minutes to one pole of the cell to undergo endocytosis (Braun et al., 1978a, 1978b; Bourguignon & Singer, 1977).

There is also good evidence that the contractile proteins are responsible for determining the shape of a cell, a property which appears to be tightly coupled to DNA synthesis and growth in normal cells (rounded cells will not proliferate while flattened or extended cells will) (Folkman & Moscona, 1978).

It has also been postulated that the cytoplasmic microfilaments play a role in transmitting signals from the cell surface, such as the binding of a lectin, to the nucleus, where the appropriate response, such as the stimulation of mitosis, would take place (Puck,

1977).

Two other protein fiber systems, the microtubules (reviewed in Cell Motility, Book C, Eds. Goldman et al., 1976) and the 100 Å filaments (Bennet et al., 1978), are found in eukaryotic cells and may often act along with the contractile proteins to determine cell shape and membrane structure (Fig. 8). There is yet limited evidence for a biochemical connection between the contractile and microtubular systems (Griffith & Pollard, 1978), but there is, in some cells (for example platelets, reviewed by White & Gerrard, 1979), very strong evidence for a functional linkage.

2. Actin and Myosin in Non-Muscle Cells

The distribution of actin in non-muscle cells has been visualized by electron microscopy as well as the use of fluorescent antibodies and fluorescent derivatives of actin (Lazarides, 1976a,b; Webster et al., 1978; Kreis et al., 1979).

In all cells actin can be found in the form of single microfilaments (about 6 nm in diameter) or joined into large networks of filaments, or aggregated side by side to form thick actin bundles or stress fibers. A characteristic property of microfilaments and the higher forms of microfilament organization is their ability to appear and disappear quickly as needed, in membrane ruffles, in pseudopods, contractile rings, or other structures.

As a cell changes shape, or proceeds through the cell cycle, it is observed that there is a constant reordering of the actin filaments. It is thought that the labile nature of the actin cytoskeleton is due in large part to a rapid interconversion between the

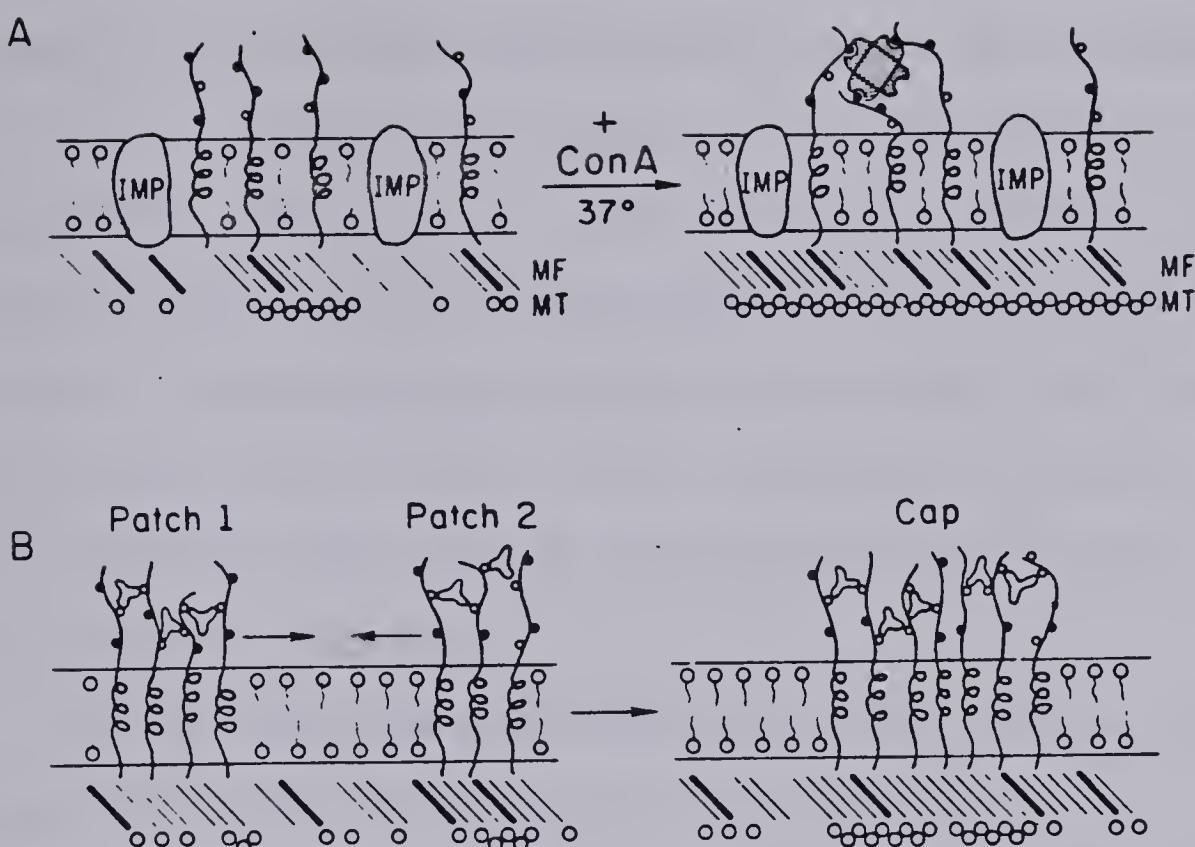


Fig. 8. Possible interactions of actin filaments (MF) with the microtubular system (MT) and membrane surface proteins. (A) Cell surface receptors penetrate the cell membrane and interact with actin microfilaments. Some of the receptors are anchored to the microtubule system through as yet unknown molecules (dark bars). Crosslinkage of certain surface protein receptors (here with Concanavalin A) could alter the various equilibria between the microfilaments, microtubules and their subunits, and induce a propagated assembly of microtubules leading to a larger proportion of anchored receptors, and affecting the structure of the cell surface. (B) Schematic illustration of the induction of caps by actin and associated contractile proteins. Patches, formed after crosslinking of specific receptors by divalent antibodies, are drawn together into one large cap by means of the contractile protein system. This process is independent of the microtubular system, although the assembly of microtubules under caps has been observed.

[from Edelman, 1976]

F and G forms of actin.

Non-muscle actins, although isolated from many different cell types, have been well characterized in only a few instances (Gordon et al., 1976a,b, 1977; Uyemura et al., 1978; Pardee & Bamburg, 1979). These studies involved actin purified from a typically diverse range of organisms: Acanthamoeba castellani (an amoeba), Dictyostelium discoideum (a slime mold), chick brain, human platelets, and rat liver. In all cases care was taken to isolate a purified actin that was representative of the major species present in the cell.

All the non-muscle actins exhibited M.Wts., binding sites for nucleotides and divalent cations, and polymerization and depolymerization properties very similar to those of muscle actin. Under physiological conditions (0.1M KCl, 1 to 2 mM Mg⁺⁺, 25 °C), non-muscle and muscle actins possessed very nearly identical critical concentrations. The results make it clear that the G to F transformations which occur in non-muscle cells do not depend on special properties of the non-muscle actins themselves.

All types of non-muscle actins are able to bind specifically to myosin and, at infinite concentrations, activate the myosin Mg⁺⁺ ATPase to the same V_{max} as skeletal muscle actin. The actins differ though in K_{app}, the concentration of actin required to activate the myosin ATPase to one half V_{max}. Actins from mammalian non-muscle cells (brain, liver and platelet) display a K_{app} 1.3 times that of muscle actin (Gordon et al., 1977), while Acanthamoeba actin (Gordon et al., 1976b) and Dictyostelium actin (Uyemura et al.,

1978) exhibit K_{app} 's 2.5 times as large.

It can be stated then that although all actins, regardless of source, are very similar, some small differences must exist among them. Isoelectric focussing has resolved three different types of actin in various mammalian cells (Whalen et al., 1976; Garrels & Gibson, 1976). A more reliable and sensitive method, that of sequence analysis, has indicated that at least six different actin genes are expressed in a single mammalian species (Vandekerckhove & Weber, 1978a). There is, in cows, one different type of actin from each of cardiac and skeletal muscle, and two different forms of actin from each of smooth muscle and non-muscle tissues. The two different non-muscle actins are highly conserved; actins from bovine brain, bovine thymus, and mouse SV40 transformed 3T3 cells appear to have identical sequences (Vandekerckhove & Weber, 1978b).

The six mammalian actins form a group of highly similar proteins, the only charged amino acid changes found among them being restricted to the first few amino terminal residues. Out of 375 residues, non-muscle and muscle actins differ in only about 25 positions, with most of these changes being conservative in nature.

The complete amino acid sequence of actin from the lower eukaryotic organism Physarum polycephalum (Vandekerckhove & Weber, 1978c), provides further evidence to indicate that actin is one of the most highly conserved proteins. Physarum actin differs from non-muscle actins in only about 17 positions, and from skeletal actin in 35 positions, with only one charged amino acid changed past the first four residues. There are no additions or deletions to the

polypeptide chain.

The fundamental functional similarities between muscle and non-muscle actins are well illustrated by the recent experiments of Kreis et al., (1979). Fluorescently labelled skeletal muscle actin, when injected into fibroblasts, entered the intrinsic actin pool and participated in the assembly and disassembly of filamentous structures.

To summarize then, it would have to be said that although small differences do apparently exist between the muscle and non-muscle actins, these differences do not, in any significant fashion, alter the basic properties of the protein.

Myosins from many non-muscle sources also appear to closely resemble the muscle protein, at least in size, shape, and subunit composition (see Table IV in Korn, 1978, and Table I in Adelstein et al., 1979). In isotonic buffers the non-muscle myosins aggregate to form bi-polar filaments, which, however, are much shorter and thinner than those formed by muscle myosin (Neidermann & Pollard, 1975). Non-muscle myosins are generally more soluble than their muscle counterparts, and there is a possibility that they undergo a reversible aggregation that can be altered depending on the particular function they are performing (Taylor et al., 1976).

As Table (I) indicates, the amount of myosin in non-muscle cells is quite low relative to the amount of actin, so that there is a very high actin to myosin molar ratio (approx. 1:100). This may be because the contractile force required in non-muscle cells is much less than that required in muscle, or because much of the

actin in non-muscle cells is either in a non-polymerized form or is used in a cytoskeletal role. The total amount of actin in a non-muscle cell may far exceed the amount which, at any one time, is involved in contractile events.

The ATPase properties of most non-muscle myosins are very similar to those of skeletal muscle myosin, with the exception that phosphorylation of one of the light chains is required for actin activation (discussed in the next section).

A completely different form of myosin has been found to exist in the amoeba Acanthamoeba. This organism contains, in addition to the more conventional two headed myosin (Myosin II) (Pollard et al., 1978; Maruta & Korn, 1977b,c), unusual single headed myosins (Myosins IA and IB) unrelated to the two headed enzyme (Maruta et al., 1978, 1979; Pollard & Korn, 1973). Myosins IA and IB contain only one heavy chain and two light chains for a total M.Wt. of 180,000, yet their Ca^{++} , K-EDTA, and Mg^{++} ATPases are very similar to those of skeletal muscle myosin. It is possible that on closer examination different forms of myosin, each perhaps involved in a different aspect of cell movement, may also be found in mammalian cells.

3. Regulation of the Actin-Myosin Interaction in Non-Muscle Cells

In skeletal muscle contraction is controlled simply by preventing the myosin from interacting with the F-actin filaments. Non-muscle actin-myosin interactions may be controlled by this mechanism as well, but it is clear that additional levels of regulation are possible. These include the transformation of myosin

from an inactive to an active form, the localization of myosin in certain parts of the cell, the depolymerization of actin filaments and their rearrangement, and the aggregation of actin filaments into bundles or gels.

The mechanism by which contraction is allowed to occur at a specific point in the cell to serve a specific function is bound to be very complex, and is not as yet understood for any non-muscle system. There is general agreement that a change in the intracellular concentration of Ca^{++} is the fundamental regulator of contraction in non-muscle cells, as it is in muscle. The possibility that other factors besides Ca^{++} could play a role in regulating the interaction of actin and myosin cannot be excluded, but most research so far has concentrated on systems which could be mediated by a rise in the concentration of Ca^{++} .

(a) Myosin Linked Regulation

Mammalian smooth muscle tissues contain quite a different sort of Ca^{++} sensitizing system than the inhibitory, thin filament linked regulatory system of skeletal muscle. Smooth muscle does not seem to contain a protein corresponding to troponin, and it is thought that the myosin is free to interact with the thin filaments at all times. However, the smooth muscle myosin is not activated by this interaction, and thus contraction is not produced, unless one of the light chains of the myosin is covalently modified by the addition of a phosphate group (Chacko et al., 1977; Small & Sobieszek, 1977; Gorecka et al., 1976).

An exactly analogous situation was shown to occur with non-

muscle myosins isolated from platelets (Adelstein & Conti, 1975), and baby hamster kidney cells (Yerna et al., 1979). In these cases phosphorylation of the 20,000 dalton light chain of the myosin stimulated the actin activation of the Mg^{++} ATPase by five to eight times.

The Ca^{++} sensitive step in this control mechanism is the phosphorylation of the myosin, which, once phosphorylated, is active regardless of the Ca^{++} concentration (Sherry et al., 1978). The kinase responsible for the phosphorylation, termed myosin light chain kinase, has been purified from smooth muscle (Dabrowska et al., 1978), platelets (Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979), and baby hamster kidney cells (Yerna et al., 1979), and in all cases is only active in the presence of Ca^{++} and the Ca^{++} binding protein calmodulin. The myosin light chain kinase and calmodulin form a tight complex in the presence of Ca^{++} , which dissociates if Ca^{++} is removed.

In the absence of Ca^{++} the kinase is inactive and the myosin is returned to its resting state by the action of a phosphatase.

That this phosphorylation reaction is important for the regulation of movement in non-muscle cells gains support from the studies of Daniel et al. (1977) on intact platelets. They found that the amount of PO_4 incorporated into the 20,000 dalton light chain of myosin increased five times when the platelets were stimulated by thrombin to change their shape and secrete substances into the surrounding medium. The time course of light chain phosphorylation was followed and was found to precede or coincide with the occurrence

of these reactions.

(b) Actin Linked Regulation

The fact that a myosin linked regulatory system exists in non-muscle cells does not rule out the possibility that regulation may also occur by means of proteins attached to the actin filaments. It is known that the muscles of most species of invertebrates contain both myosin and troponin linked regulatory systems (reviewed by Kendrick-Jones & Jakes, 1976).

There is, as well, a fundamental difference between the myosin light chain kinase regulatory system, which activates an inactive state in the presence of Ca^{++} , and the troponin-Tm thin filament type of system, which inhibits an active state in the absence of Ca^{++} . It could well be an advantage for a non-muscle cell to have both mechanisms of control, if, for example, the time course for dephosphorylation of the myosin was much longer than the time required to once again lower the Ca^{++} concentration.

A number of attempts have been made to demonstrate thin filament regulation in non-muscle cells, and although a Tm like protein has been found, the search for a troponin like component has been less successful.

Several reports have appeared suggesting that partially purified non-muscle actins have proteins bound to them with the ability to confer Ca^{++} sensitivity to the interaction between actin and myosin. Both crude platelet actin, contaminated with proteins of 36,000, 30,000, 18,000, and 14,000 daltons (Cohen et al., 1973),

and crude brain actin, contaminated with proteins of 38,000, 30,000, 27,000, and 20,000 daltons (Puszkin & Kochwa, 1974) were claimed to be able to render a muscle myosin preparation Ca^{++} sensitive. The M.Wts. of the actin associated proteins are of course suggestive of the presence of components equivalent to muscle Tn-T, Tm, Tn-I, and Tn-C. Similar results have been reported for a crude preparation isolated from the slime mold Physarum (Kato & Tonomura, 1975; Nachmias, 1975). In no case, however, have the Ca^{++} sensitizing proteins been purified and characterized.

Mahendran and Berl (1977, 1979) were able to isolate a fraction from brain consisting of three proteins (36,000, 18,700, and 14,000 daltons) which, when mixed with brain Tm and skeletal muscle actin and myosin, produced a small degree of Ca^{++} sensitivity. The brain fraction was able to bind to a G-actin affinity column and could be separated into its constituent components by chromatography on a DEAE Sephadex column in the presence of 6M urea. The isolated components exhibited activities in a skeletal actomyosin ATPase assay consistent with the proposition that they were equivalent to skeletal muscle Tn-T, Tn-I, and Tn-C; however, the brain proteins eluted from the DEAE column in a different order, and had different M.Wts. than the muscle troponin subunits. The very small amounts of brain "troponin" which could be isolated made the characterization of the protein difficult, and necessitated the use of an ATPase assay which could easily have produced erroneous results.

Condeelis and Taylor (1977) have made the interesting proposal

that the Ca^{++} sensitivity seen in these crude fractions is actually due to a gel to sol transformation of the F-actin (discussed in the next section). However, because the components of these studies have been so poorly characterized, it is at present impossible to predict the mechanism by which they are acting.

Although Tn-I and Tn-T like proteins have never been convincingly demonstrated to exist outside of skeletal and cardiac muscle, the same cannot be said of Tn-C. A low M.Wt. Ca^{++} binding protein, with 50% direct sequence homology to Tn-C (Dedman et al., 1978) can be isolated from virtually all eukaryotic cells. This protein, calmodulin (also known as Ca^{++} dependent regulator protein, modulator protein and protein activator), is present in large amounts in certain non-muscle cells (90 mg per kg in brain, Sharma et al., 1979) and regulates a large number of cellular functions, often by forming Ca^{++} dependent complexes with enzymes, thereby activating them.

It has already been mentioned that calmodulin is known to be involved in the regulation of the actin-myosin interaction in non-muscle cells through its association with the myosin light chain kinase enzyme, but it is tempting to speculate that it also performs a Tn-C like function as part of a thin filament regulatory system. If this is the case the other components of such a system (which may bear no resemblance to Tn-I and Tn-T) could perhaps most easily be found by isolating proteins which are able to bind to calmodulin. Quite a number of such proteins have been recently identified (Sharma et al., 1978, 1979; Grand & Perry, 1979), but none have yet

been shown to interact with actin.

Calmodulin is, to an extent, able to replace Tn-C in its interactions with the other troponin components. Calmodulin can form a complex with Tn-I which, however, is dissociated either in the absence of Ca^{++} or in the presence of 5M urea, conditions under which the Tn-I: Tn-C complex is stable (Amphlett et al., 1976; Grand et al., 1979). Calmodulin is also able to bind to Tn-T (Amphlett et al., 1976; Dedman et al., 1977), and thus can be used to prepare a hybrid troponin (calmodulin:Tn-I:Tn-T) (Dedman et al., 1977; Fine et al., 1975).

The Ca^{++} sensitivity obtained with the calmodulin troponin in a skeletal Tm, actomyosin system is quite poor, because it appears that the lower affinity of calmodulin for Tn-I prevents a complete neutralization of inhibitory activity in the presence of Ca^{++} (Fig. 9).

Cohen and Cohen (1972) were the first to isolate a protein resembling muscle Tm from a non-muscle cell, the platelet. The purified protein had an α -helix content greater than 90% and an amino acid composition very similar to muscle Tm. The major difference was that the non-muscle protein had a smaller subunit M.Wt. than the muscle Tm (30,000 as opposed to 35,000). In addition, paracrystals formed of the platelet Tm gave an axial repeat of 34.3 ± 5 nm, significantly shorter than that found for skeletal Tm (39.5 nm).

Proteins with structural characteristics similar to Tm have since been isolated in small amounts from brain (Fine et al., 1973), sea urchin eggs (Ishimoda-Takagi, 1978), pancreas and fibroblasts (Fine & Blitz, 1975). All of these non-muscle Tms were of

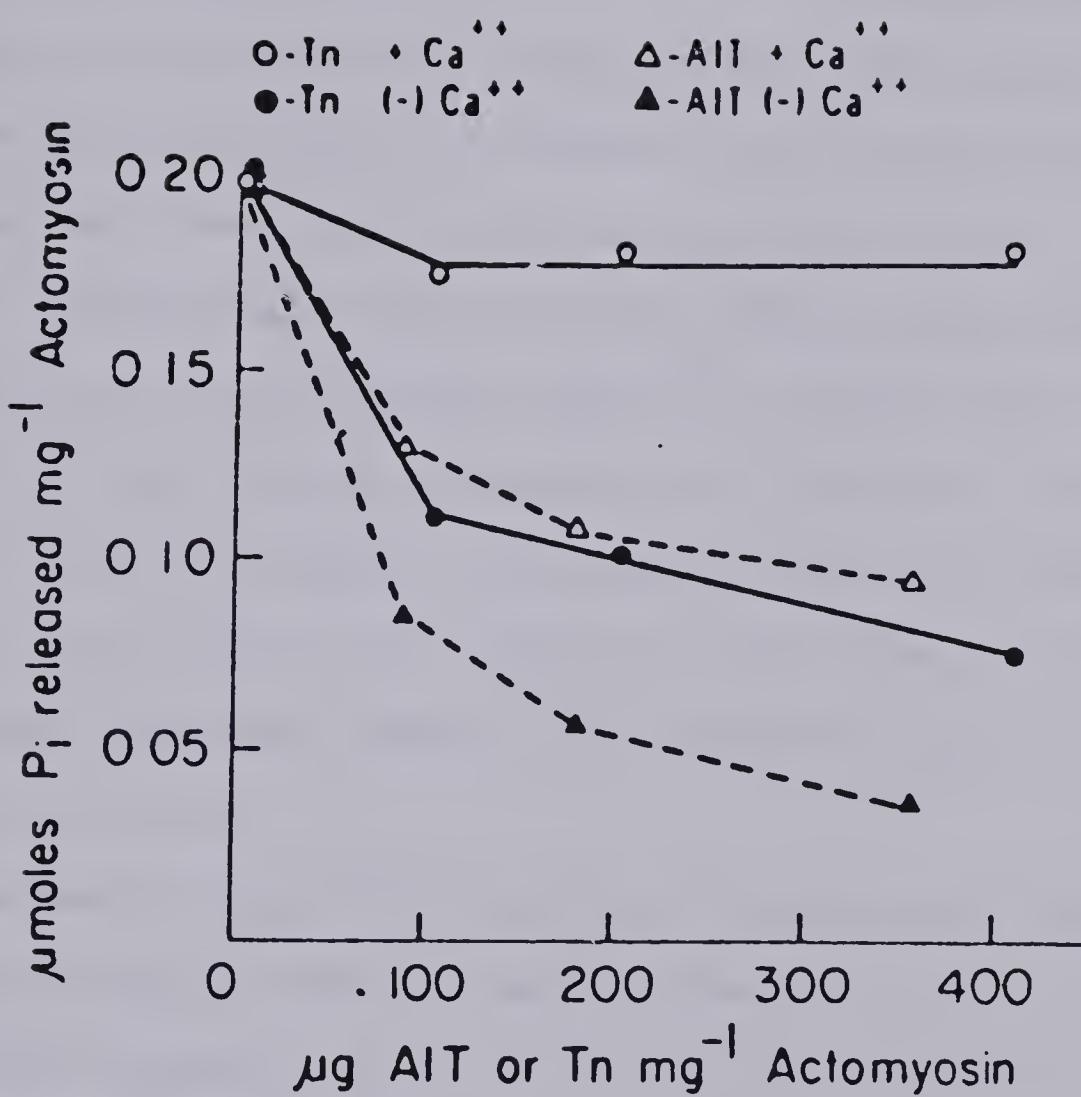


Fig. 9. Regulation of rabbit skeletal actomyosin by a calmodulin-Tn-I-Tn-T complex (AIT) and rabbit skeletal troponin (Tn). Actomyosin ATPase assays were conducted in a 5 mM Tris, pH 7.5 buffer, containing 25 mM KCl, 1 mM MgCl₂, 1 mM ATP and 0.48 mg of actomyosin. For each concentration of AIT or Tn an equal amount (wt/wt) of Tm was included. The symbols signify the following: o, Tn + 0.1 mM CaCl₂; o, Tn + 1 mM EGTA; △, AIT + 0.1 mM CaCl₂; ▲, AIT + 1 mM EGTA. [from Dedman et al., 1977]

the lower M.Wt. variety, indicating that perhaps there are two distinct classes of Tm, a longer form found only in muscle tissues (skeletal, smooth and cardiac), and a smaller form found in non-muscle cells. Evidence that non-muscle Tms from quite distinct sources (calf brain, pancreas and platelets) are in fact very similar comes from the work of Fine and Blitz (1975), who found that these three proteins produced identical peptide maps after digestion with chymotrypsin.

The localization of Tm in non-muscle cells grown in tissue culture has been studied by Lazarides using immunofluorescent techniques with antibodies prepared against the skeletal muscle protein. He finds that Tm is only associated with actin filaments that are organized into large bundles, or stress fibers (Lazarides, 1976a). Such stress fibers are thought to have mainly a structural function and are most often seen in fully spread out cells, where they are associated with the plasma membrane that contacts the substratum (Goldman et al., 1976)

Treatment of spread fibroblasts with trypsin causes them to round up and the stress fibers to rapidly dissociate. At this stage the actin and Tm appear to exist in a diffuse form throughout the cell. Many cells, such as lymphocytes, exhibit this pattern of staining all the time. When replated, the fibroblasts start to slowly spread out, and distinct actin bundles once again become visible, but now they are organized into a highly regular polygonal array with α -actinin bound at the vertices of the network (Lazarides, 1976a,b) At first Tm is not found to be associated with this network, but after a considerable lag time it can again be seen bound

to the actin bundles (Lazarides, 1976a,b).

In general, the fibroblast Tm always appears to be absent from highly mobile and dynamic structures, where presumably the actin filaments are undergoing continuous changes in their organization. Because of this, Lazarides (1976a) suggests that Tm is not involved in either the initiation or assembly of actin filaments, or in the regulation of motility in dynamic areas such as membrane ruffles. Tm may confer structural stability to actin filaments engaged in maintaining cell shape.

Lazarides (1975) has also observed that while stress fibers reacted with actin specific antibody exhibit a continuous fluorescence along their lengths, those reacted with anti-Tm antibody display a periodic fluorescence. The fluorescent segments vary from 0.8 to 1.7 μm in length (average 1.2 μm), while the non-fluorescent spacings in between are 0.3 to 0.5 μm long. Antibodies to α -actinin produce a periodic fluorescence with segment lengths the inverse of those seen for Tm (Lazarides & Burridge, 1975). If the fibers are stained with both anti-Tm and anti- α -actinin antibodies a continuous fluorescence is observed, implying that lying along the actin bundles are alternate regions of bound Tm and bound α -actinin.

Other proteins involved in contraction such as calmodulin and myosin have also been found by immunofluorescent techniques to be specifically associated with the actin stress fibers (Dedman et al., 1978; Heggeness et al., 1977; Weber & Groeschel-Stewart, 1974). In fact, the changes in distribution of myosin during the cell cycle have been shown to parallel exactly the changes in the localization of

actin (Pollard et al., 1976).

(c) Regulation by Actin Assembly

Actin can be present in a non-muscle cell either as monomeric G-actin, as F-actin filaments, as networks or gels of F-actin filaments, or as highly ordered actin filament bundles. The transformation between one state of actin and another is controlled by a number of proteins, more of which are being discovered all the time. Since all of these proteins bind to actin it is likely that they will, in one way or another, influence the interaction of Tm with actin, and consequently the ability of Tm to function in a thin filament regulatory system. As has been previously mentioned, immunofluorescence studies lend support to the notion that the role of Tm in non-muscle cells is closely tied to the organizational state of the actin. In addition, it is clear that the assembly and disassembly of actin filaments is, in itself, a powerful method by which the interaction of actin and myosin may be controlled.

Proteins that control the assembly of actin can be divided into two groups, 1) those that prevent the polymerization of G-actin to F-actin, and 2) those that crosslink F-actin filaments to form gels or bundles.

The presence of the first group of proteins was suspected because various cell extracts contained high concentrations of unpolymerized actin under conditions where the pure actin would have formed filaments. G-actin from spleen that would not polymerize under a variety of conditions was found to be bound in a tight 1:1 complex with a small (16,000 dalton) basic protein which was called

profilin (Carlsson et al., 1976, 1977). Profilin has also been identified in brain, thymus, and lymphocyte cells (Carlsson et al., 1977), as well as platelets (Markey et al., 1978). A protein which appears to be very similar to profilin has been isolated from Acanthamoeba (Reichstein & Korn, 1979).

It is estimated that there is enough profilin in platelets to inhibit the polymerization of at least 55% of the total platelet actin, and at least 75% of the polymerization resistant actin that can be extracted from platelets is bound to profilin (Markey et al., 1978).

By utilizing another protein, DNase I, which can not only form a tight 1:1 complex with actin (or actin-profilin), but can also depolymerize F-actin (Lazarides & Lindberg, 1974; Hitchcock et al., 1976), an assay has been developed to determine the amounts of unpolymerized actin in cell extracts (Blikstad et al., 1978; Markey et al., 1978). The results indicate that 50 to 75% of platelet actin, 50 to 55% of HeLa cell actin, and 60 to 65% of lymphocyte actin is present in the cell in an unpolymerized form.

No specific mechanism has yet been found to dissociate the profilin-actin complex, although it appears that one must be present in order to free the actin to participate in contractile events. Surprisingly, the very tight DNase I-actin complex can be separated into its two components by the plasma membrane bound enzyme 5' nucleotidase (Mannherz & Rohr, 1978; Rohr & Mannherz, 1979). It is not yet known if the interaction of DNase I with G-actin is of widespread occurrence, or of physiological significance, even though Rohr & Mannherz (1978) have been able to demonstrate the natural

occurrence of the complex in rat pancreatic juice.

Controlled, reversible actin filament assembly could conceivably play an important role in the regulation of non-muscle cell motility, for contraction can only take place if actin filaments are available. Profilin and DNase I are probably only a small part of a complex system governing where and when actin filaments will form.

Besides being found as single filaments, F-actin in crude cell extracts often aggregates either to form bundles (0.1 to 0.2 μm in diameter), or gels. Both of these supramolecular forms of actin appear transiently during the cell cycle, so that controls must exist to regulate their formation in response to appropriate stimuli.

The binding of actin filaments together into large bundles may impart to the filaments a strength and rigidity which, individually, they would be lacking. Such a structure may enable actin to better perform a cytoskeletal role; for example, actin bundles form the long spiky pseudopods of platelets and the microvilli of intestinal cells, as well as the stress fibers which appear to be responsible for the flattening or elongation of tissue culture cells.

By aligning the microfilaments into parallel arrays the ability to produce force in conjunction with myosin may be optimized. Such a case may occur during cell division, where the actin becomes organized into one large bundle just beneath the plasma membrane (the contractile ring) (Schroeder, 1975).

Very highly ordered actin bundles can be formed by a 58,000 dalton protein (58k protein) isolated from sea urchin eggs (Bryan & Kane, 1978). Using optical diffraction and image reconstruction

techniques it was found that these bundles were composed of parallel, exactly aligned actin filaments arranged in a hexagonal lattice with a spacing of 8.3 nm (Fig. 10) (DeRosier et al., 1977; Spudich & Amos, 1979). Cross-bridges between actin filaments are formed by the 58k protein and appear to involve two actin monomers on each filament. Because not all actin monomers are exactly aligned, some cross-bridges are strained or distorted and can take up either of two possible positions (dotted lines in fig. 10).

DeRosier et al. (1977) believe that the 58k protein occupies the myosin binding site on actin and may be involved in the regulation of the actin-myosin interaction. No experimental results are available to indicate whether or not these bundles can activate the Mg^{++} ATPase of myosin, or bind to T_m .

Many extracts of cells form thick gels when warmed to room temperature. Under the electron microscope, these gels appear as amorphous aggregates probably produced by a random cross-linking of actin filaments. Proteins capable of forming actin gels range from the low M.Wt. (23,000 to 38,000) gelactins isolated from Acanthamoeba, to filamin, a protein from smooth muscle composed of two identical 250,000 dalton subunits (Wang, 1977; Wang & Singer, 1977).

Filamin is closely related to an actin binding protein (ABP) from mammalian non-muscle cells, which has so far been isolated from macrophages (Hartwig & Stossel, 1975; Stossel & Hartwig, 1976a) and is known to be present in platelets and fibroblasts (Wallach et al., 1978). Antibodies to filamin cross-react with ABP and

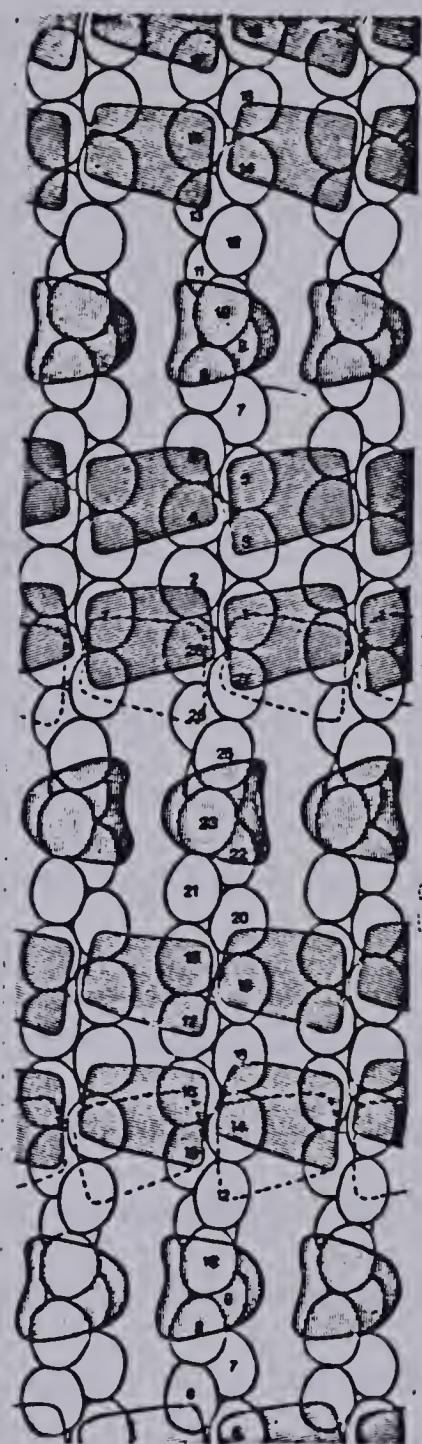


Fig. 10. Schematic drawing, produced from computationally filtered electron micrographs, showing the probable arrangement of actin filaments and cross-bridges in actin bundles from the microvilli of sea urchin eggs. The cross-bridges, formed by the 58k protein repeat at intervals of about every 13.0 nm, corresponding to a spacing of four or five actin subunits. Each cross-bridge interacts with four F-actin subunits. Some cross bridges appear able to take up two possible positions (dotted lines). In the drawing 1 cm corresponds to 8.0 nm.
[from Spudich & Amos, 1979]

can be used to show that in non-muscle cells ABP is associated with actin bundles and with a diffuse intracellular matrix of single F-actin filaments (Heggeness et al., 1977).

It requires only one molecule of filamin per 190 molecules of actin, or one ABP per 740 molecules of actin, to form an actin gel, so that these proteins can be effective in very small amounts (Brotschi et al., 1978).

Gels prepared from crude cell extracts can often be broken down to sols, that is free F-actin filaments, by the addition of micromolar Ca^{++} , or by small changes in pH (7.0 to 7.4) (Brotschi et al., 1978; Bryan & Kane, 1978; Condeelis & Taylor, 1977). A protein (actinogelin) capable of forming a Ca^{++} sensitive actin gel has been purified from Ehrlich tumour cells (Mimura & Asano, 1979).

Condeelis and Taylor (1977) postulate that gel to sol transformations play an important role in the Ca^{++} regulation of contraction. They have shown, using extracts from amoeba, that the actin-myosin interaction leading to contraction is prevented when the actin is in the form of a gel. Purified proteins which form gels of F-actin solutions (gelactins and filamin) are able to inhibit the actin activated ATPase of myosin (Maruta & Korn, 1977a; Davies et al., 1977).

C. AIMS OF THIS PROJECT

The fact that contractile proteins perform a wide range of important functions in all eukaryotic cells has only recently been realized. Much of the research in this field over the last several years has had to be devoted to the task of proving that actin, myosin, and other muscle proteins do exist in most cells and tissues. Now that this task is largely completed, the emphasis in research has shifted, and is now concentrated on the search for, and isolation of, novel non-muscle proteins, perhaps with no counterpart in the muscle system, which may be involved in contractile events.

Eventually, in order to fully understand the role played by each of these new proteins in cell motility, it will be necessary to study, on a biochemical and molecular level, their structures, functions, and interactions with each other. We have attempted to characterize in this manner a protein, Tm, which evidence (as outlined in the introduction) suggests is involved with contractile events in non-muscle cells. Very few non-muscle contractile proteins have been studied in detail, mainly it would appear, as a result of the difficulty in purifying sufficient amounts of them. The first aim of this project was therefore to determine the feasibility of isolating large amounts of a non-muscle Tm in a pure form.

This was shown to be possible and two methods were developed for purifying Tm from platelets (chosen as the source material for reasons given in Chapter III). This provided a base for all further work to be performed with the protein, the ultimate goal of which

will be the full understanding of the role played by Tm in the process of cell motility. At present such a goal is unattainable, since so many of the proteins with which platelet Tm may interact are poorly characterized or, perhaps, even undiscovered. The aims of this project were then to characterize the non-muscle Tm structurally, by physical and chemical methods, and functionally by obtaining information on its ability to interact with the skeletal muscle contractile proteins. It was hoped that the results of such studies would provide an insight into the manner in which Tm may act in non-muscle cells.

Most of the experiments in this thesis involved a comparison between the properties of skeletal muscle α Tm and platelet Tm. By attempting to relate the differences observed to alterations in the sequences or structures of the two proteins, it was hoped that some information on the particular regions or features of skeletal α Tm responsible for its ability to bind troponin and actin, and to function in the regulation of skeletal muscle contraction, would be obtained.

CHAPTER II

MATERIALS AND METHODS

A. PHYSICAL METHODS

1. Gel Electrophoresis

SDS gel electrophoresis was performed on slab gels, containing 8 to 10% acrylamide, using either the system of Weber and Osborn (1969) or of Laemmli (1970). SDS gels were sometimes run in the presence of 6M urea (Sender, 1971).

For samples containing many different proteins greater resolution of the various bands could be achieved using gels containing a gradient of acrylamide, from 5% at the top to 20% at the bottom. Gradients were formed by an apparatus consisting of two plastic graduated cylinders connected to each other by tubing fitted into holes drilled into each cylinder near the base. Another piece of tubing, leading from a second hole at the bottom of the cylinder containing the 20% acrylamide mixture, carried the gel solution to the gel apparatus. The gel solution in this case contained only one half the normal amount of ammonium persulfate to ensure that polymerization did not occur too quickly.

Samples for all gels were made up in a solution consisting of 6M urea, 50 mM PO₄, and 1% SDS. Subsequently, mercaptoethanol (1%) and Bromphenol blue (1% of a 0.5% solution in 0.1M PO₄) were added to each sample, and the samples then incubated for 1 hour at 60°C.

After electrophoresis, gels were washed in 10% methanol, 10%

acetic acid for at least 2 hours, stained with Coomassie Brilliant Blue R-250 (0.25% solution in 50% methanol, 10% acetic acid) for 1/2 hour, then destained in 10% methanol, 10% acetic acid overnight or longer.

Where necessary, after complete destaining gels were sliced into strips and scanned at 596 nm on a Gilford spectrophotometer with gel scanning attachment and recorder.

2. Circular Dichroism

(a) Determination of α -Helical Contents

Circular dichroism (CD) studies were performed on a Cary model 60 recording spectropolarimeter with a Cary model 6001 CD attachment according to the procedure described by Oikawa et al. (1968).

The mean residue ellipticity, $[\Theta]_{\lambda}$ (in $\text{deg} \cdot \text{cm}^2 \cdot \text{dmole}^{-1}$) at a given wavelength, λ , was calculated using the equation

$$[\Theta]_{\lambda} = \Theta_{\text{obs}} / 10 \ell c \quad (1)$$

where; m = mean residue weight (taken as 115)

ℓ = the cell path length in cm

c = the protein concentration in g/ml

Θ_{obs} = the ellipticity measured at λ

The CD spectra of any protein can be expressed, at any wavelength λ , by the relationship

$$[\Theta]_{\lambda} = f_H [\Theta]_{H\lambda} + f_{\beta} [\Theta]_{\beta\lambda} + f_R [\Theta]_{R\lambda} \quad (2)$$

where f_H , f_{β} , and f_R represent the fractional values of α -helix, β -sheet, and random coil structure in the protein ($f_H + f_{\beta} + f_R = 1$), while $[\Theta]_H$, $[\Theta]_{\beta}$, and $[\Theta]_R$ are the mean residue ellipticity reference values for the three types of secondary structure (Chen et al., 1974).

With the assumption that T_m contains no regions of β -sheet structure, equation (2) reduces to

$$f_H = \frac{[\Theta]_L - [\Theta]_{R\lambda}}{[\Theta]_{H\lambda} - [\Theta]_{R\lambda}} \quad (3)$$

This relationship, along with the following parameters of Chen et al. (1974), was used to determine the percent α -helix in T_m .

$$\begin{aligned} [\Theta]_R &= 1,580 \text{ at } 222 \text{ nm, } -2,200 \text{ at } 210 \text{ nm} \\ [\Theta]_H &= -39,500 \text{ at } 222 \text{ nm, } -38,500 \text{ at } 210 \text{ nm} \end{aligned}$$

Circular dichroism data is sometimes reported in terms of the difference in the extinction coefficients of the sample for left and right circularly polarized light,

$$\Delta\epsilon = \epsilon_L - \epsilon_R$$

The molar ellipticity $[\Theta]_M$ is related to $\Delta\epsilon$ by the expression

$$[\Theta]_M = 3305 [\Delta\epsilon] \quad (\text{Adler et al., 1973})$$

$[\Theta]_M$ is calculated using the M.Wt. of the protein in equation (1) in place of the mean residue weight.

(b) Melting Temperature Measurements

CD measurements were used to determine the melting temperature of T_m . The temperature of the protein solution was raised in a step-wise fashion using a water jacketed cell compartment and Lauda Thermo-regulator. Ellipticity values were read at 221 nm 15 minutes after the raising of the temperature to ensure that the sample had reached

thermal equilibrium. The reversibility of the denaturation process was followed by measuring the ellipticity values as the temperature was lowered in a stepwise manner.

3. Ultracentrifugation

Ultracentrifugation studies were performed at 20°C on a Beckman Spinco Model E analytical ultracentrifuge using a Rayleigh interference optical system. Sedimentation equilibrium runs used the methodology of Richards et al. (1968).

The apparent weight average molecular weight, M_w , determined by sedimentation equilibrium is given by

$$M_w = \frac{2 RT}{(1 - \bar{v}\rho) \omega^2} \cdot \frac{d\ln c}{dr^2}$$

where; R = the gas constant (1.987 cal/deg.mole)

 T = the experimental temperature in °K

\bar{v} = the partial specific volume of the protein

ρ = the solvent density

ω = the angular velocity in radians/second

 c = the protein concentration at a distance r,
 from the axis of rotation

4. Absorbance Spectrophotometry

Routine absorbance measurements were performed using a Gilford 240 spectrophotometer. Ultraviolet absorbance spectra were recorded on a Cary 118c recording spectrophotometer.

The presence of tryptophan was determined in 0.1N NaOH using the method of Goodwin and Morton (1946) as described by Donovan (1969). The number of moles of tyrosine and tryptophan per mole of protein

can be determined from the molar extinction coefficients (ϵ_{λ}) at different wavelengths

$$M_{Tyr} = 10^{-3}(0.592 \epsilon_{294.4} - 0.263 \epsilon_{280.0})$$

$$M_{Trp} = 10^{-3}(0.263 \epsilon_{280.0} - 0.170 \epsilon_{294.4})$$

5. Viscosity Measurements

Viscosity experiments were performed using a Cannon-Manning semi-micro type A50 viscometer, requiring a 0.5 ml charge, and with a flow through time for water of about 5 minutes. Samples, dissolved up in and dialyzed overnight against the appropriate buffer, were rendered dust free by passage through a 10 to 20 μm pore size sintered glass filter, and then diluted to the desired concentration with the dialysate prior to determination of their viscosities at room temperature. All buffers contained 10 mM cacodylate pH 7.0, 2 mM mercaptoethanol, and NaCl to give the required ionic strength.

In the experiments involving both troponin and Tm, the two proteins were dialyzed separately, then mixed to achieve the required final concentrations of each. The solutions were allowed to sit at room temperature for an hour before their viscosities were determined.

In all cases the samples were run through the viscometer at least twice with the average time being taken to determine η_{rel} .

Between runs, the viscometer was rinsed out with H_2O , then with acetone and air dried on a water pump. At the end of each day the viscometer was cleaned with chromic acid.

6. Preparation and Electron Microscopy of Paracrystals

Paracrystals of platelet and skeletal Tm were grown by a method adapted from that of Caspar et al. (1969). The proteins were dissolved in 1.0M KCl, 50 mM Tris, pH 8.0, at a concentration of 5 mg/ml, centrifuged to remove dust, and then dialyzed against 50 mM MgCl₂, 50 mM Tris, pH 8.0, at 4°C. After three days the samples were removed from the dialysis bag and the paracrystals collected by centrifugation in a bench top centrifuge.

Samples were examined in a Philips EM 300 electron microscope, after being placed on carbon coated grids and negatively stained with an aqueous 1% uranyl acetate solution. Photographs were taken at an enlargement of 48,000 x.

B. CHEMICAL METHODS

1. Amino Acid Analysis

(a) General

Amino acid analyses were performed on a Durrum model D-500 or a Beckman model 120c analyzer. Both machines automatically integrated the peak areas of the amino acids.

The samples to be hydrolyzed were dissolved in constant boiling HCl with 0.1% phenol, evacuated and sealed, and then incubated at 110°C for 24 hours. The tubes were subsequently cracked open and dried down over NaOH pellets in a desiccator. For quantitative determinations of certain amino acids it was necessary to incubate the samples for varying lengths of time (usually 24, 48,

and 96 hours). For the amino acids isoleucine and valine the longest time values were used, while for threonine and serine the values were obtained by extrapolation back to zero time.

(b) Cysteic Acid

Cysteine was determined after conversion to cysteic acid by the method of Moore (1963). Protein used for this procedure was first dialyzed for one day against 4 mM mercaptoethanol and then for three days against 10^{-3} M HCl.

Samples, hydrolyzed as for a normal amino acid analysis, were run on the Beckman analyzer. Concentrated samples were used to give accurate values for cysteic acid, then carefully diluted ten times and rerun to yield values for alanine and leucine, by means of which the protein concentration could be calculated. The colour value of aspartic acid was used for cysteic acid.

2. Determination of Protein Concentration

(a) Absorbance Measurements

Protein concentrations of pure samples were usually determined by means of the absorbance at 280 nm (A_{280}) using extinction coefficients given in the literature (Table II). Concentrations in mg/ml were converted to molarity using the M.Wts. listed in Table (II).

(b) Dye Binding Method

During the various stages of purifying platelet Tm total protein contents were determined by the Coomassie Brilliant Blue G-250 dye binding method of Bradford (1976).

Protein solutions containing 5 to 40 μ g protein in a volume

TABLE II
Extinction Coefficients and M.Wts.

Protein	$E_{280}^1 \text{ mg/ml}$	Refer- ence	M.Wt.	Refer- ence
calmodulin	0.18	a	18,000	a
actin	1.11	b	42,000	c
Tn-I	0.596	d	21,000	e
Tn-C	0.193	d	18,000	f
Tn-T	-	-	31,000	g
troponin	0.47	h	70,000	(sum of parts)
S-1	0.79	i	115,000	j
cardiac Tm (skeletal α Tm)	0.33	k	66,000	m
platelet Tm	0.24	k	57,000	k

a) Watterson et al. (1976)

b) Houk and Ue (1974)

c) Elzinga et al. (1973)

d) Margossian and Cohen (1973)

e) Wilkinson and Grand (1975)

f) Collins et al. (1973)

g) Pearlstone et al. (1977)

h) Lovell and Winzor (1977)

i) Yagi et al. (1967)

j) Weeds and Taylor (1975)

k) This study

m) Stone et al. (1974)

of 50 μ l were pipetted into test tubes containing 1.5 ml of protein reagent. Standard curves were prepared using bovine serum albumin or actin. Both proteins yielded very similar results.

Samples which would not dissolve up in H_2O (ex. freeze dried platelets) were solubilized in 0.1N NaOH for five minutes at 100°C prior to determination of their protein content (Chiappelli et al., 1979).

(c) Amino Acid Analysis

Amino acid analysis was often used to determine the concentrations of pure proteins with known sequences. For this purpose the values obtained for alanine and leucine, corrected by means of standards, were used. Protein concentration can then be calculated from the M.Wt. of the protein, the number of moles of alanine or leucine per mole of protein, and the volume of sample loaded onto the analyzer.

Protein concentrations of impure fractions were calculated by adding up the molar values of all amino acids, multiplying by the average amino acid M.Wt. (usually 115), and then correcting for the volume of sample loaded onto the analyzer.

3. Determination of the Extinction Coefficient

Amino acid analyses were used to determine $E_{280}^{1\%}$ values for α skeletal and platelet Tm. The absorbance of protein solutions was determined at 280 nm, samples were taken in triplicate by means of Accupetts (Dade) or Lamda pipets (Fisher), hydrolyzed, dried down, and redissolved in an accurately measured volume of pH 2.2 citrate

buffer before being loaded onto the amino acid analyzer. Protein concentrations were calculated from the alanine and leucine values, knowing that platelet Tm contains 30 alanine and 30 leucine residues and that skeletal α Tm contains 36 alanine and 33 leucine residues.

The calculated protein concentrations were plotted against the measured A_{280} values to yield a straight line, the slope of which, determined by least squares analysis, yielded $E_{280}^{1\%}$.

4. Gel Filtration

Bio-Gel A-1.5m was packed into a column (0.9 x 110 cm) and equilibrated at 5 ml/hour with buffer containing 1.0M NaCl, 1 mM DTT and 10 mM Tris, pH 8.0. Protein samples were dialyzed overnight against the column buffer and centrifuged prior to being applied to the column. Fractions were collected in volumes of 20 drops. The tube containing the peak concentration of the protein was taken as being the elution volume, V_e . V_o , the void volume, was determined using Blue Dextran, and V_t , the total accessible column volume, by using NaCl. The NaCl elution position was detected using a conductivity meter (Radiometer).

Data was treated as described by Mann and Fish (1972), by normalizing the solute elution position in terms of the distribution coefficient K_d . K_d is determined from the following relationship

$$K_d = \frac{V_e - V_o}{V_t - V_o}$$

Under the non-denaturing conditions used the elution volume of a protein is governed not by its M.Wt., but by its Stokes radius

(the radius of the equivalent hydrodynamic sphere). However, for most globular proteins a straight line relationship can be obtained by plotting Kd vs log M.Wt.

5. Sequence Methods

High voltage electrophoresis of peptide mixtures, and manual sequence analysis by the dansyl-monitored Edman degradation were performed as described by Hodges and Smillie (1972).

6. Protein Purification

All skeletal muscle proteins were prepared from frozen rabbit muscle (Rabbit muscle tissue, Type I, mature, New Zealand White) purchased from Pel-Freeze. The muscle tissue, protected inside a plastic bag, was thawed out under cold running water just before use.

(a) Troponin

Troponin was prepared by the method of Staprans et al. (1972) with the modification that centrifugation at 4,200 rpm for 10 minutes in an IEC PR-6000 centrifuge replaced extraction through cheesecloth as the means to separate supernatant and muscle residue. The final pH 4.5 precipitation of Staprans et al. (1972) was repeated, after which the supernatant was adjusted to pH 7.6 with KHCO₃ and fractionated by the addition of solid ammonium sulfate. Precipitated material was collected at 45, 50, 55, and 60% saturation (at 0°C). These were taken up in H₂O and dialyzed against 2 mM mercaptoethanol before freeze drying.

Troponin was separated into its components by the method of Greaser and Gergely (1971).

CB-1, a fragment of Tn-T, was prepared by Dr. J. Ng following the method of Pearlstone et al. (1975).

(b) Actin

Actin acetone powder was prepared by the method of Carston and Mommaerts (1963), except that one ethanol extraction (3 volumes) was performed before the acetone extractions. The acetone powder, stored at -20°C, appeared to be stable indefinitely.

The procedure of Spudich and Watt (1971) was used to prepare actin from the powder. The only modification was that the extract, containing actin, was separated from the muscle residue by filtration through a thick paper pulp, formed from well swollen and shredded Whatman #1 paper. The filtrate was always clear at this stage, obviating the need for centrifugation.

The final purified G-actin (about 4 mg/ml) was stored at 4°C and used within two weeks of preparation.

Actin was prepared from fresh horse platelets as described by Gordon et al. (1977) for the purification of human platelet actin. This procedure is patterned on an earlier method for purifying actin from Acanthamoeba (Gordon et al., 1976a)

(c) Myosin S-1

Thawed rabbit skeletal muscle (100g), was cut into small pieces and blended in a Waring blender for 15 seconds in 100 ml of Guba-Straub buffer (0.3M KCl, 2.5 mM MgCl₂, 1 mM EGTA, 0.15M KH₂PO₄, pH 6.5). The mince was added to 600 ml of the same buffer containing 1.2 mM ATP, extracted for 15 minutes with stirring at 4°C, and centrifuged. The supernatant was diluted with 9 liters of cold

H_2O to precipitate myosin, which was then collected by centrifugation and redissolved in 60 ml of concentrated (5 times) Guba-Straub buffer containing 5 mM ATP. The final volume was made up to 300 ml with H_2O . The resulting solution was clarified at 80,000 times gravity for 3 hours, filtered through Whatman #54 paper, and precipitated again by addition to 10 liters of H_2O . The myosin, recovered by centrifugation, was redissolved in 60 ml of 1.1M KCl, 5 mM EDTA, 0.75M KH_2PO_4 , pH 7.0 buffer, immediately precipitated by addition to 2.5 liters of H_2O , and then taken up in 250 ml of 0.5M KCl, 0.3M Tris, 1 mM EDTA, pH 7.0 buffer. After overnight dialysis against this buffer the myosin solution was clarified at 80,000 times gravity for 2 hours, then poured slowly into an equal volume of saturated ammonium sulfate, pH 7.0 (previously treated with 4g of Chelex 100 per liter (Bio Rad)). This yielded a gelatinous white precipitate, which, after centrifugation, was dissolved in as small a volume as possible (about 100 ml) of 1.0M NaCl.

S-1 was cleaved from the myosin as described by Weeds and Taylor (1975). After the chymotryptic digestion had been stopped by the addition of 0.5 mM PMSF, the solution containing S-1 was immediately centrifuged to remove insoluble material. The supernatant was dialyzed against 40 mM Tris, 1 mM EDTA, pH 7.4, any further precipitate removed by centrifugation, and the sample loaded onto a DEAE cellulose column (Whatman DE-52). The column (2.5 x 25 cm), previously equilibrated with 40 mM Tris, 1 mM EDTA, pH 7.4, was eluted with a linear gradient (1 liter) of KCl to 0.2M.

S-1 eluted in two peaks, corresponding to protein containing the A-1 or A-2 light chain. Both forms of S-1 have essentially the

same activity under the conditions in which the ATPase assays described in this thesis were performed (Wagner et al., 1979), and so they were pooled together. The S-1 solution was concentrated, using a 500 ml Amicon concentrator fitted with a PM-10 membrane, to a concentration of 3 to 4 mg/ml. S-1 could be stored at 4°C for at least six weeks without loss of activity.

The essential sulfhydryl groups of S-1 were always reduced before use by dialyzing the S-1 against buffer containing 2 mM DTT. To check the activity of the enzyme the K^+ EDTA ATPase (in 0.6M KCl, 5 mM EDTA, 2 mM DTT, 2 mM ATP, pH 8.0) was measured. Activities of 3.5 to 4.0 μ moles PO_4^{2-} /min/mg S-1 ($6.7 \text{ to } 7.7 \text{ sec}^{-1}$) were generally obtained, close to published values for S-1, which vary from 7.3 to 11.2 sec^{-1} (Margossian et al., 1975; Margossian & Lowey, 1978)

(d) Muscle Tm

Cardiac and skeletal muscle Tm were purified by the method of Pato (1978). Cardiac Tm was prepared from rabbit hearts (Type I, New Zealand White, mature; Pel-Freeze).

The two forms of skeletal Tm, α and β , were separated under denaturing conditions (8M urea) by the method of Cummins and Perry (1973). However, instead of carboxymethylating the Tm, 5 mM DTT was added to all buffers so that the sulfhydryl groups of the Tm would remain reduced. Only the α form of rabbit skeletal Tm was used for experiments.

At times cardiac Tm was used in place of rabbit skeletal α Tm. Amino acid sequence analysis has demonstrated that these two proteins are identical (Mak et al., 1979). Cardiac Tm was used in preference

to skeletal α Tm in many biological experiments, since cardiac Tm, during its preparation, had not been exposed to denaturing conditions. In general, however, the two proteins could be used interchangeably.

Cardiac Tm preparations were sometimes contaminated with nucleotides (producing a low A_{280}/A_{260} ratio), which could be removed by passage through a DEAE cellulose column (Hodges, 1972).

e. Calmodulin

Calmodulin was prepared in our laboratory by R. Bergstrom from bovine brains, following the procedure of Walsh (1978).

7. Iodination of Tm

Cardiac and platelet Tms were iodinated with $Na^{125}I$ (carrier free, 17 Curies/mg, 50 mCuries/ml; New England Nuclear) by the lactoperoxidase method following the procedure of Eaton et al. (1975). The precautions suggested by Morrison (1974) were followed; 5 μl (0.25 mCuries) of $Na^{125}I$ (in 0.1N NaOH) was neutralized with 2.5 μl 1M KPO_4 , pH 7.0, and reduced with 7.5 μl of 10 μM sodium sulfite (in order to convert any iodine present to iodide), after which 12 μl (0.2 mCuries) were added to a solution containing 15 mg of Tm and 25 μg of lactoperoxidase (lyophilized powder; Sigma Chemical Co.) in 2 ml of 0.4M KCl, 50 mM KPO_4 , pH 7.0.

The reaction was initiated by the addition of 5 μl of 0.03% H_2O_2 ; further aliquots were added every 10 minutes. Following the fourth aliquot 5 μl of 5 mM KI was added, then, 10 minutes later, a final 5 μl of 0.03% H_2O_2 was added. After a further 10 minutes

the reaction was terminated with 20 μ l of 50 mM DTT.

The 125 I-labelled Tm was dialyzed against 0.1M KCl, 1mM DTT until the dialysate contained less than 30,000 cpm/ml. The 125 I-Tm was stored in solution at 4°C, and its concentration determined by amino acid analysis. When counted in Aquasol (New England Nuclear) using a liquid scintillation counter (Beckman LS-230) the specific activity of the platelet Tm used in this thesis was 3.12×10^6 cpm/mg, while that of the cardiac Tm was 9.75×10^6 cpm/mg, when initially prepared.

C. OTHER METHODS

1. Platelet Counts

Samples of plasma, diluted 10 to 20 times with 0.1M formaldehyde, were placed into a Hemacytometer counting chamber (AO Spencer, "Bright Line", Improved Neubauer), and platelets and red blood cells counted using phase contrast microscopy (Carl Zeiss, Ph2 Neofluar 25 objective). Five squares, 0.2 mm x 0.2 mm x 0.1 mm, were counted for each sample, the average taken, and the number of cells per cubic mm calculated.

2. Actin Binding Studies

Binding studies were based upon the co-sedimentation methods of Laki et al. (1962), Hitchcock et al. (1973), and Eaton et al. (1975). Reagents and protein solutions were mixed in small centrifuge tubes at room temperature (final volume of 1 ml), allowed to stand with occasional shaking for 15 minutes, and then centrifuged for two hours at 35,000 rpm at 20°C using a 50 rotor in a Beckman

L2-65 B ultracentrifuge. Prior to use in the assay all protein solutions, except actin, were dialyzed overnight against the assay buffer and centrifuged in the 50 rotor at 35,000 rpm for one hour.

In experiments with a constant Mg⁺⁺ concentration all proteins, except actin, were dialyzed against buffer containing the appropriate amount of Mg⁺⁺. In experiments where the Mg⁺⁺ concentration was varied, MgCl₂ was added directly to each centrifuge tube from stock solutions.

The amount of Tm bound to actin was determined by two methods.

(a) Binding Determined by Gel Scanning

After centrifugation the actin pellet in each tube was washed, without disturbing the pellet, with 1 to 2 ml of H₂O, and then dissolved in about 1 ml of SDS urea sample buffer before being electrophoresed on SDS gels. After staining and destaining, the gels were scanned at 596 nm and the area under the actin and Tm peaks measured with a planimeter (Keuffel and Esser).

The relationship between staining intensity and protein concentration became non-linear if the maximum absorbance of the actin peak increased over 1.5, or the absorbance of the Tm peak dropped below 0.2. Care was taken to dissolve the actin pellet up in the appropriate amount of SDS urea buffer so that the sizes of the protein peaks would fall within these limits, although where the level of Tm bound to actin was small this was not possible.

(b) Binding Determined by Radioactivity

This method depends on determining the radioactivity in a

given volume of solution before and after centrifugation (Eaton et al., 1975). The decrease in the number of cpm following centrifugation can be directly related to the amount of Tm which has been bound to actin, and thus removed from the supernatant. Controls indicated that in the absence of actin no platelet Tm, and very little cardiac Tm, sedimented.

A correction was performed to account for the percentage of actin (usually 5 to 10%) which did not sediment. Samples were taken from a control tube (containing only actin) before and after centrifugation, and the amount of protein in each sample determined from a standard curve prepared for actin using the dye binding method of Bradford (1976).

Three 100 μ l samples were usually taken before and after centrifugation from each centrifuge tube, added to 10 ml of Aquasol, and counted in a Beckman LS-230 liquid scintillation counter, using the ^3H and ^{14}C windows. Knowing the specific activity of the Tm, and the amount of actin which had sedimented, the difference in counts could be used to calculate the molar ratio of Tm to actin in the pellet.

A problem was encountered, however, in that the cpm recorded for each sample continually decreased with time (about 5% per day). Heating the samples in 3% SDS, 3% mercaptoethanol before adding them to the Aquasol (in order to reduce aggregation) as recommended by Yang et al. (1977) did not eliminate the problem. Samples placed directly into glass test tubes and counted using a γ -ray counter (LKB 1270 Rackgamma II), gave results which corresponded

very closely to the results obtained by liquid scintillation counting, providing that the Tm samples were counted immediately after addition to the Aquasol. Therefore, these counts were the ones used for determining the amount of Tm bound to actin.

Corrections were applied to account for the decay of the ^{125}I , which has a half-life of 60.2 days (therefore a decrease in cpm of 0.9885 per day).

3. ATPase Assays

ATPase assays were performed by the pH-stat method using a Radiometer TTT-2 Titrator, SBR2c Titrigraph, and SBULa syringe burette. This method is dependent on the fact that hydrolysis of ATP to ADP + PO_4^{2-} releases hydrogen ions. The resultant drop in pH, detected by a pH electrode (Radiometer GK2321c) immersed in the reaction solution, is automatically corrected for by the Titrator, which acts to release KOH from the syringe burette into the reaction solution. The Titrigraph records the distance moved by the syringe plunger as a function of time. For these experiments, each 0.1 inch division on the chart equaled a 0.25 mm movement of the plunger, which resulted in a 5 μl aliquot of KOH being released from the 1.0 ml syringe (Agla, micrometer all glass syringe; Burroughs Wellcome and Co., England).

A KOH stock solution (0.4 to 0.5M), standardized against KH Phthalate (Fischer Primary Standard), was used to prepare the dilute KOH solutions (10 to 20 mM) needed for the ATPase assays.

The apparent pK of the reaction measured, $\text{H}_2\text{PO}_4^- \rightleftharpoons \text{HPO}_4^{2-} + \text{H}^+$, was determined by use of the pH-stat; a constant amount of

ATP was added to an actin-S-1 system and titrated to the end point at various pHs. By measuring the amount of KOH consumed at each pH the pK of the reaction being measured was calculated to be 6.7. From this value, the amount of KOH of known molarity added could be converted into ATPase activity in terms of μ moles PO_4 /minute released.

Assay samples, 2 ml in volume, were placed in glass vials and continuously stirred throughout the experiment by means of a small teflon coated bar and magnetic stirring motor. The temperature was held constant at 25°C through use of a glass water jacket and Haake circulating water bath.

The actin activated ATPase of S-1 was measured, unless otherwise stated, in 30 mM KCl, 0.1 mM EGTA, 5 mM MgCl_2 , 2 mM DTT, 2 mM disodium ATP, and 2 mM Tris. The pH was held at a constant value of 7.8 by the pH-stat.

All proteins, except actin, were dialyzed overnight against the assay buffer (minus the ATP) prior to use in the assay. Troponin was found to be more soluble in this low ionic strength buffer if dissolved up first in buffer containing 0.13M KCl without EGTA. Troponin was then dialyzed to low ionic strength against the standard buffer (30 mM KCl), again in the absence of EGTA. The solubility of Tn-I, which is poor at low ionic strength (Greaser & Gergely, 1973; Horwitz et al., 1979), can be increased by lowering the pH. For this reason Tn-I was dialyzed against assay buffer at pH 6.0 before use in the assay.

ATP was added to the assay buffer from a stock 0.2M solution

prepared freshly each day by dissolving disodium ATP (Terochem Laboratories Ltd., Edmonton) in 0.5M KOH until the pH was 7.5. The ATP was then diluted to a concentration of 0.2M by the addition of assay buffer. ATP was added to each assay prior to the addition of S-1.

Ca^{++} , when needed, was added from a stock 20 mM solution of CaCl_2 to yield a final concentration in the assay of 0.2 mM.

Actin, stored at 4°C in the G form, was converted to the F form by dilution with assay buffer at least one hour before commencing ATPase assays. F-actin was always added first to the assay sample, followed, if need be, by other proteins, then by ATP. The pH was then adjusted to 7.8 by the pH-stat and the reaction initiated by the addition of S-1.

CHAPTER III

PURIFICATION OF TM FROM PLATELETS

Non-muscle Tm was first isolated, from platelets, by Cohen and Cohen (1972), and later, by basically the same method, from brain (Fine et al., 1973). In order to obtain a preparation with a high degree of purity it was necessary to elute and renature the platelet protein from an 8M urea polyacrylamide gel (Cohen & Cohen, 1972). Yields were therefore very low and extensive characterization of the protein could not be performed. The purpose of this study was to develop a method whereby large amounts of a non-muscle Tm could be isolated, allowing a number of biochemical studies, including the amino acid sequence analysis of the protein, to be undertaken.

A. PLATELETS AS THE SOURCE MATERIAL

Blood platelets were regarded as constituting the best source of a mammalian non-muscle Tm. Platelets are small cells, 2-4 μm in diameter, which circulate in the blood as smooth biconcave discs. They are formed from the cytoplasm of huge polyploid cells, megakaryocytes, found in the bone marrow, and contain no nuclei.

Platelets are mainly involved in hemostasis and when activated, for example by regions of damaged blood vessel wall or by thrombin, they undergo a dramatic shape change, involving the formation of pseudopods and the development of an irregular or spherical shape. Platelets also have the ability to secrete various substances from

intracellular storage granules and are responsible for clot retraction, which converts loosely clumped platelets and fibrin into tightly packed masses sealing the sites of vascular injury. The platelet is dependent on contractile forces to drive most of the processes which are essential to its biological function, and as a consequence is a richer source of non-muscle contractile proteins than any other mammalian cell type.

Platelets are also easy to isolate and can be prepared as a homogeneous fraction. Although some contamination with erythrocytes usually does occur, this can, if necessary, be kept to a minimum. Virtually all other mammalian non-muscle sources from which contractile proteins can be isolated in reasonable yields are composed of a heterogeneous mixture of different cell types, including smooth muscle, which are difficult or impossible to separate from each other. Uncertainty then prevails as to which cell type a given purified product is derived from, particularly if the final yield of the desired protein is very small in relation to the amount of starting material.

Since it was expected that a non-muscle T_m , even in platelets, would constitute only a very small fraction of the total cellular protein, it was essential to be sure that large amounts of the source material would be readily available. This prerequisite was met by platelets since blood was continually available to us in unlimited quantities.

The final reason for our choice of platelets as a starting material was that, in 1975 when this work was begun, platelets

were one of the few cells in which the presence of a non-muscle Tm had been conclusively demonstrated (Cohen & Cohen, 1972).

B. PREPARATION OF PLATELETS

Blood (50 liters) was collected directly from freshly killed horses into a large plastic pail containing 4 liters of anti-coagulant (60g sodium citrate· $2\text{H}_2\text{O}$, 21g citric acid· H_2O , 30g dextrose per liter, pH 5.0). The blood was immediately transported to the laboratory and allowed to settle at 4°C.

Horse erythrocytes have a high rate of sedimentation (69 to 127 mm/h) (Altman & Diltmer, 1961), so that after two hours 20 to 25 liters of plasma per pail, containing approximately 2.5×10^4 red blood cells and 3×10^5 platelets per mm^3 , could be siphoned off. Before being allowed to settle, horse blood was found to contain about 9×10^6 erythrocytes and 3×10^5 platelets per mm^3 .

To further reduce the number of erythrocytes contaminating the platelets the plasma was centrifuged at 550 times gravity for 12 minutes. Two large centrifuges, an MSE-LR6 capable of spinning 6 liters at a time, and an IEC PR-6000, also with a capacity of 6 liters, were operated simultaneously during this procedure. After this step there remained approximately 2.8×10^3 red blood cells and 2.4×10^5 platelets per mm^3 of plasma.

During the initial phase of this study platelets were collected by passing the plasma, at 350 ml/h, through a continuous flow centrifuge (Lourdes Beta Fuge, CF-1 rotor) operating at 10,000 rpm. To ensure that a high percentage of the platelets were not lost this process was performed twice. Following the second centrifugation only about 1.2×10^4 platelets remained in the supernatant.

It was later found that a faster and more convenient method to pellet the platelets was to centrifuge them at 5,000 times gravity for 12 minutes using two Sorvall RC-3 centrifuges, each with a capacity of 4 liters. Yields were higher by this method, probably because the platelets were not subjected to the high shear forces encountered during continuous flow centrifugation and so were much more likely to remain in an intact and undamaged state.

After each centrifugation the packed platelets were gently suspended in 0.15M NaCl, 10 mM sodium citrate, 1 mM EDTA, 1 mM DTT, pH 6.9 and kept at 4°C. The final suspension (about 2 liters in volume from 70 liters of plasma) was centrifuged at 5,000 times gravity for 12 minutes, the supernatant discarded, and the platelets resuspended in H₂O and lyophilized. The platelets at this stage were reddish due to slight contamination with erythrocytes (approximately 1 per 100 platelets). Attempts were not usually made to completely remove red blood cells as their presence did not interfere with the subsequent isolation of platelet Tm.

The entire procedure, from collection of blood to freezing of the platelets, was performed in one day. Yields were approximately 2.5g wet weight of platelets (0.5g lyophilized weight) per liter of plasma or 2.5 liters of blood.

C. PURIFICATION OF PLATELET TM

Tm has no easily measurable biological activity by which the amount present in a given preparation can be determined. The only method which was available to analyze the efficiency of a given

purification step was SDS gel electrophoresis. This method depended on the knowledge that the M.Wt. of platelet Tm was close to 30,000 (Cohen & Cohen, 1972), and was successful because of the fact that, although Tm is only a minor component of platelets, it electrophoresed in a region of the gel free of major protein bands. This allowed reasonable estimates to be made not only of the yields at various stages, but of the amount of Tm originally present in intact platelets.

1. Amount of Tm in Platelets

For this study platelets were washed until completely free of red blood cells and plasma. They were then lyophilized and stored in this manner until, just prior to application to an SDS gel, they were dissolved in SDS gel sample buffer and placed in a boiling water bath for 5 minutes.

The samples were electrophoresed on SDS gels which contained a concentration gradient of acrylamide, from 5% at the top of the gel to 20% at the bottom. Very high M.Wt. proteins, which would be excluded from ordinary 8% acrylamide gels, were able to enter the gradient gels, and, as well, the gradient gels were capable of retaining low M.Wt. molecules that might have electrophoresed out of ordinary gels. A more accurate estimate of a samples total protein content (by spectrophotometrically scanning the stained gel at 596 nm) could then be obtained with use of the gradient gel.

Gradient gels also produced sharper bands and provided a higher degree of resolution than ordinary gels. A typical trace of a sample of whole platelets run on an SDS gradient gel is shown in

Fig. 11. The peak which co-electrophoresed with a sample of pure platelet T_m is indicated.

As will be described in more detail later in this chapter and in Chapter IV platelet T_m, when run on SDS gels containing 6M urea, splits into two bands both of which move with a reduced mobility relative to other proteins. On SDS urea gradient gels one band of pure platelet T_m co-electrophoresed with the tail end of the large actin peak, while the other band moved with a mobility similar to that of the small peak immediately following actin (Fig. 12).

Thus it would be expected that the peak corresponding to platelet T_m on SDS gels would not be present in the same position on SDS urea gels. It can be seen that the peak designated as platelet T_m in Fig. 11 has largely disappeared in Fig. 12, confirming the identity of this band. Many of the other differences in band distribution between Fig. 11 and Fig. 12 are a result of a variation in the gradients of acrylamide for the two gel systems. The addition of 6M urea to both the 5% and 20% acrylamide solutions raises the density of both to such an extent that uniform gradients of acrylamide are difficult to produce.

The areas of the actin peak, platelet T_m peak and total protein were measured with a planimeter from scans of SDS gradient gels to give the ratios shown in Table (III). Values were determined using two baselines as indicated in Fig. 11, to yield the maximum and minimum amounts of platelet T_m that could be present. Comparison of Fig. 11 to Fig. 12 indicates that the smaller value is probably more reasonable since the baseline in this region of the gel remains

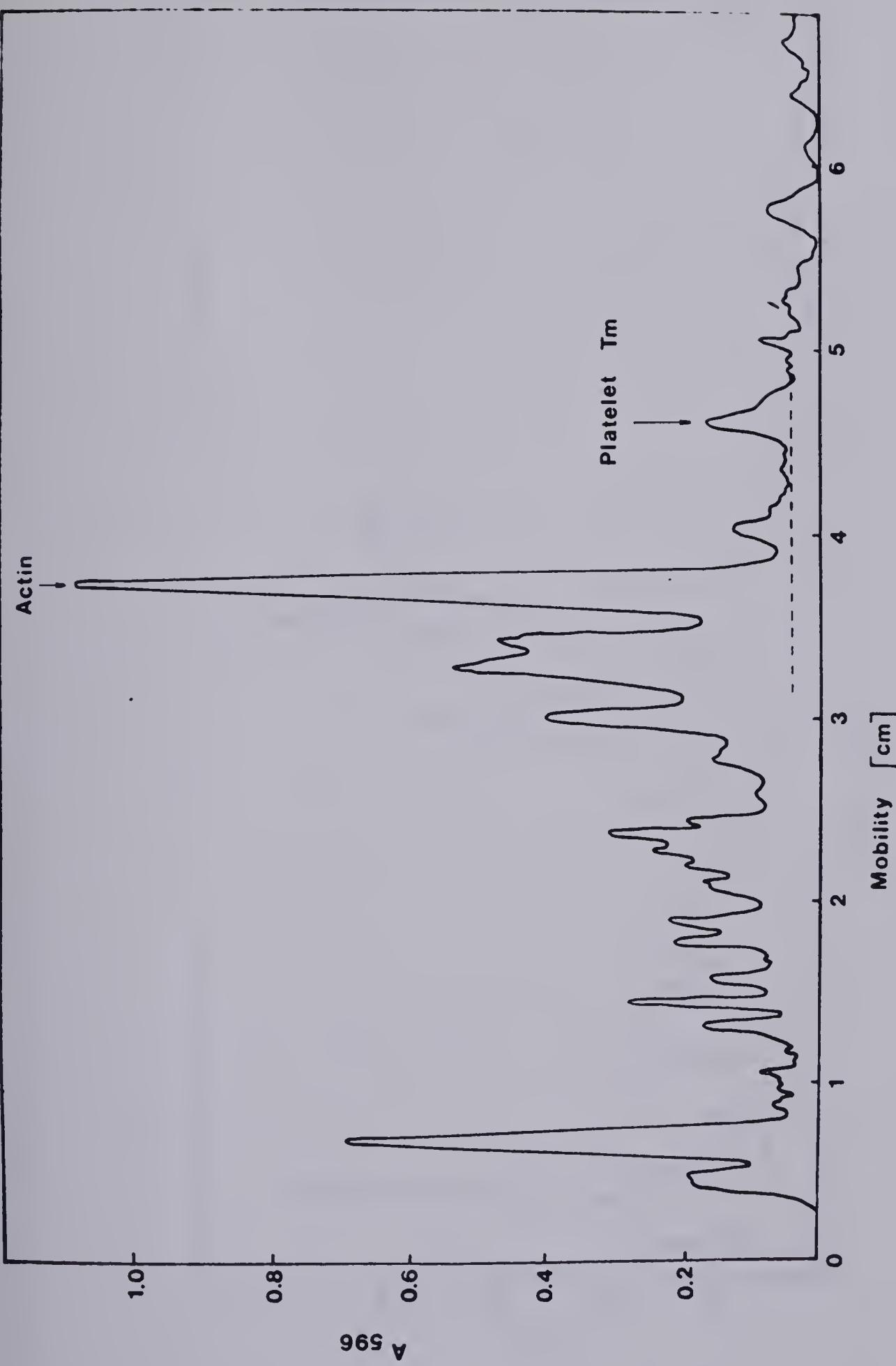


Fig. 11. Gel scan, at 596 nm, of whole platelets electrophoresed on an SDS gradient gel (5 to 20% acrylamide). The gel system was that of Weber and Osborn (1969). The peaks which co-electrophoresed with pure samples of actin and platelet Tm are indicated. The dotted line below the actin and Tm peaks represents Baseline 2, while the solid line forming the lower edge of the figure represents Baseline 1. In order to determine their area, the actin and Tm peaks were extrapolated downwards to meet the baselines (Table III). The total protein area was always determined using Baseline 1.

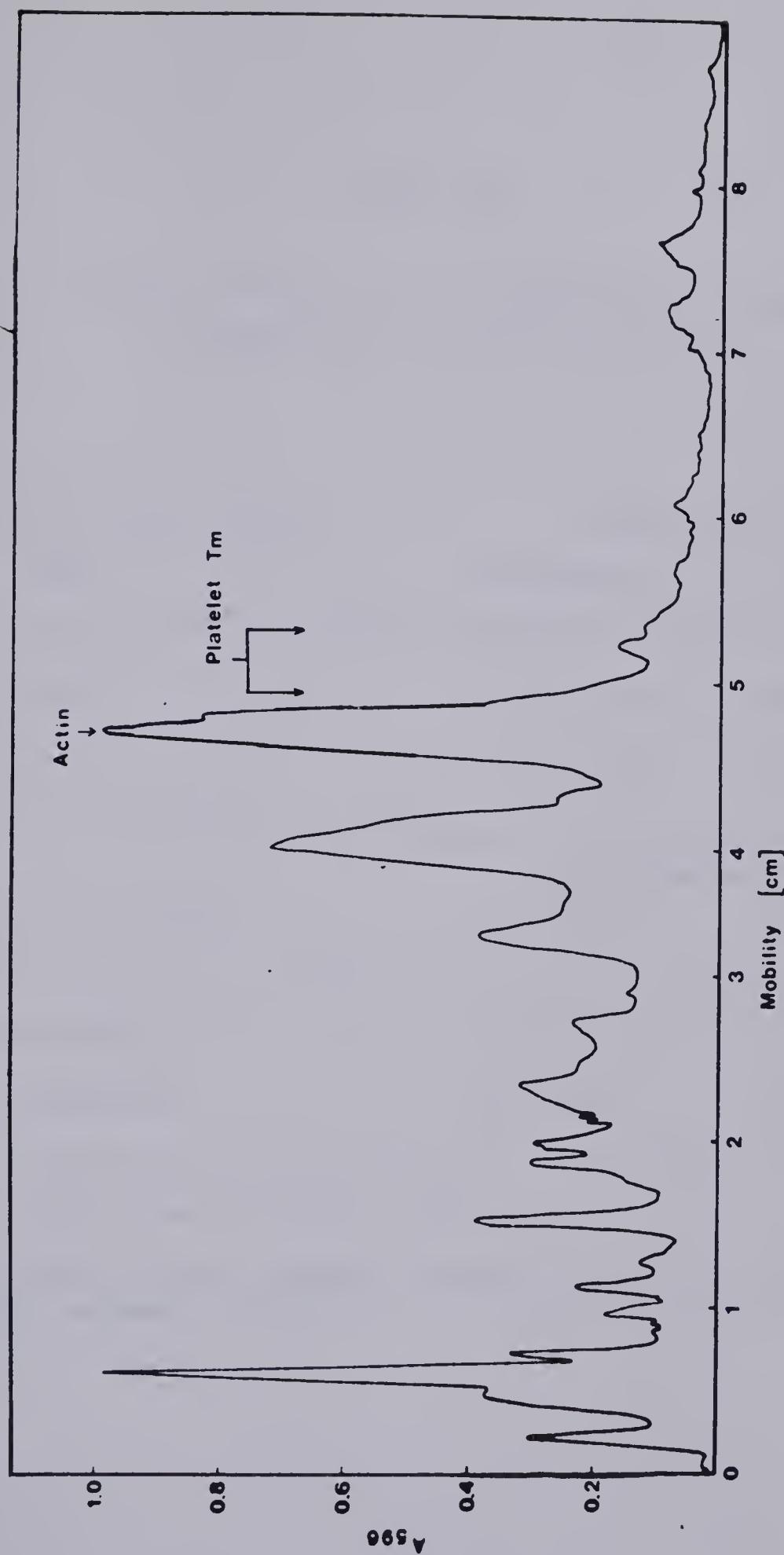


Fig. 12. A gel scan, at 596 nm, of whole platelets electrophoresed on SDS urea gels containing a 5 to 20% gradient of acrylamide. The mobilities with which pure samples of actin and platelet Tm electrophoresed are indicated. In this gel system (Sender, 1971), platelet Tm splits into two bands (see Fig. 21).

TABLE III

Amounts of Tm and Actin in Platelets as Measured
by Densitometry of Stained SDS Gels

Percent of Total Protein which is	as Determined from	
	Baseline 1 ^a	Baseline 2 ^a
Actin ^b	18.2 ± 1.0	16.4 ± 1.0
Tm	2.7 ± 0.3	1.6 ± 0.2
as Determined from		
Ratio of Actin:Tm	Baseline 1	Baseline 2
By weight	6.7 ± 1.5	10.3 ± 2.0
By molarity	9.1 ± 2.0	13.5 ± 2.5

^a The baselines used are shown in Fig. 11.

^b Values shown are the mean and standard deviation for determinations from four separate SDS gels

slightly elevated even after the platelet T_m peak has moved from the vicinity. This means that there is on the order of one T_m molecule for every thirteen actin molecules in platelets, and that T_m composes about 1.5% of the total platelet protein.

These figures can only be approximations as a result of the differential staining of various proteins by the dye (Coomassie Blue) and of the difficulty in determining the true baseline.

2. Extraction of T_m from Platelets

Skeletal T_m is prepared from an acetone powder of muscle. The ethanol and acetone washes remove membranes, denature many of the myofibrillar proteins and allow increased amounts of T_m to be extracted (Bailey, 1948). Cohen and Cohen (1972) in isolating a non-muscle T_m followed this procedure by preparing an acetone powder of platelets.

At first the acetone powder method was used in this study; however, it was abandoned when it was discovered that T_m could be extracted directly from the washed platelets. The amounts that could be extracted were greater if the platelets were first ruptured by lyophilization or homogenization, and the yields were better than could be obtained by extraction from an acetone powder.

3. Purification Method 1

This was the first method of purification devised for platelet T_m and was used in preparing all of the protein needed for sequence work and much of the protein used in physical, chemical and biological studies. A flow diagram of the procedure is shown in Fig. 13 while Table (IV) summarizes the amount of protein and T_m present at the

10g Platelets (lyophilized)

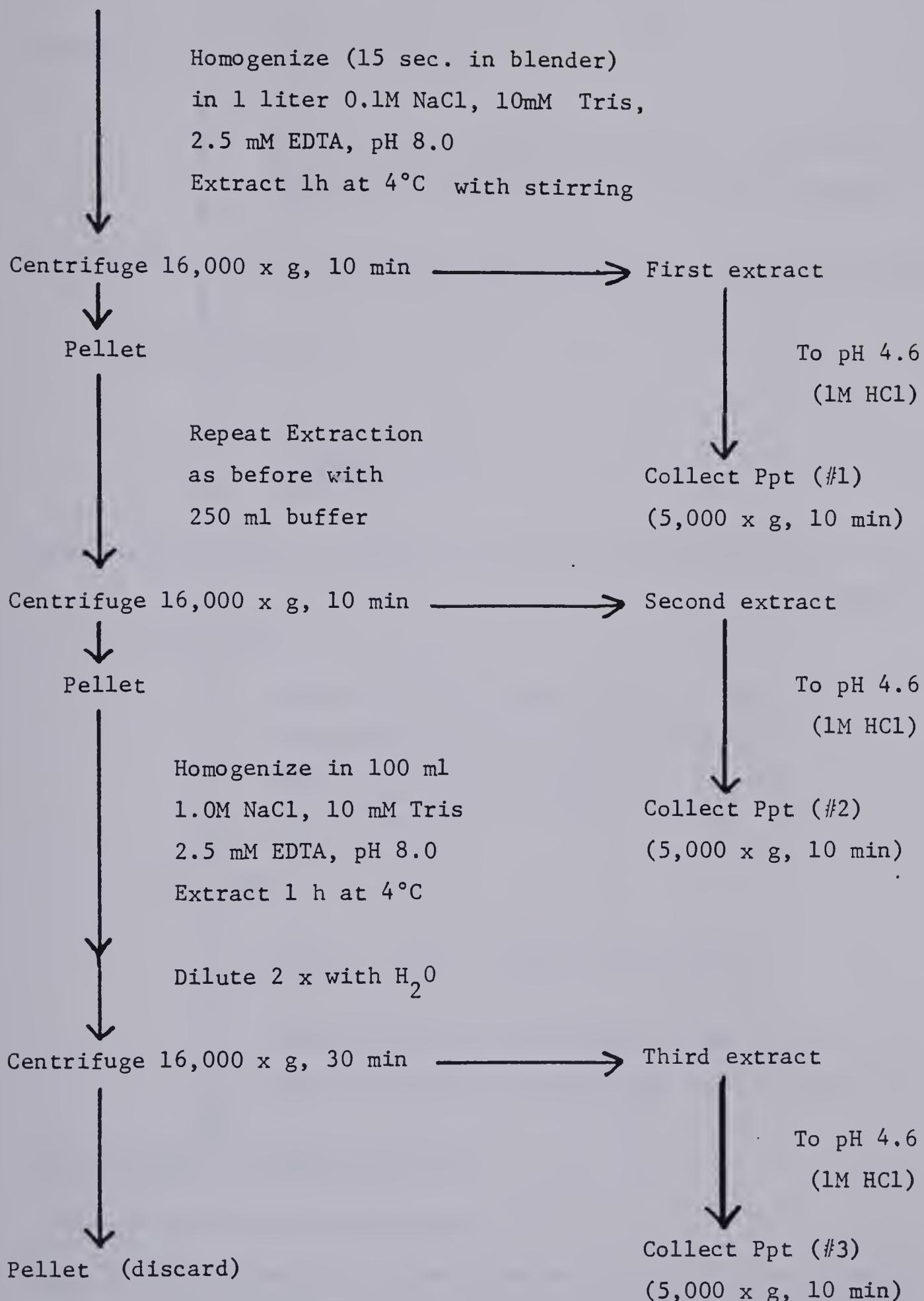


Fig. 13 (continued next page).

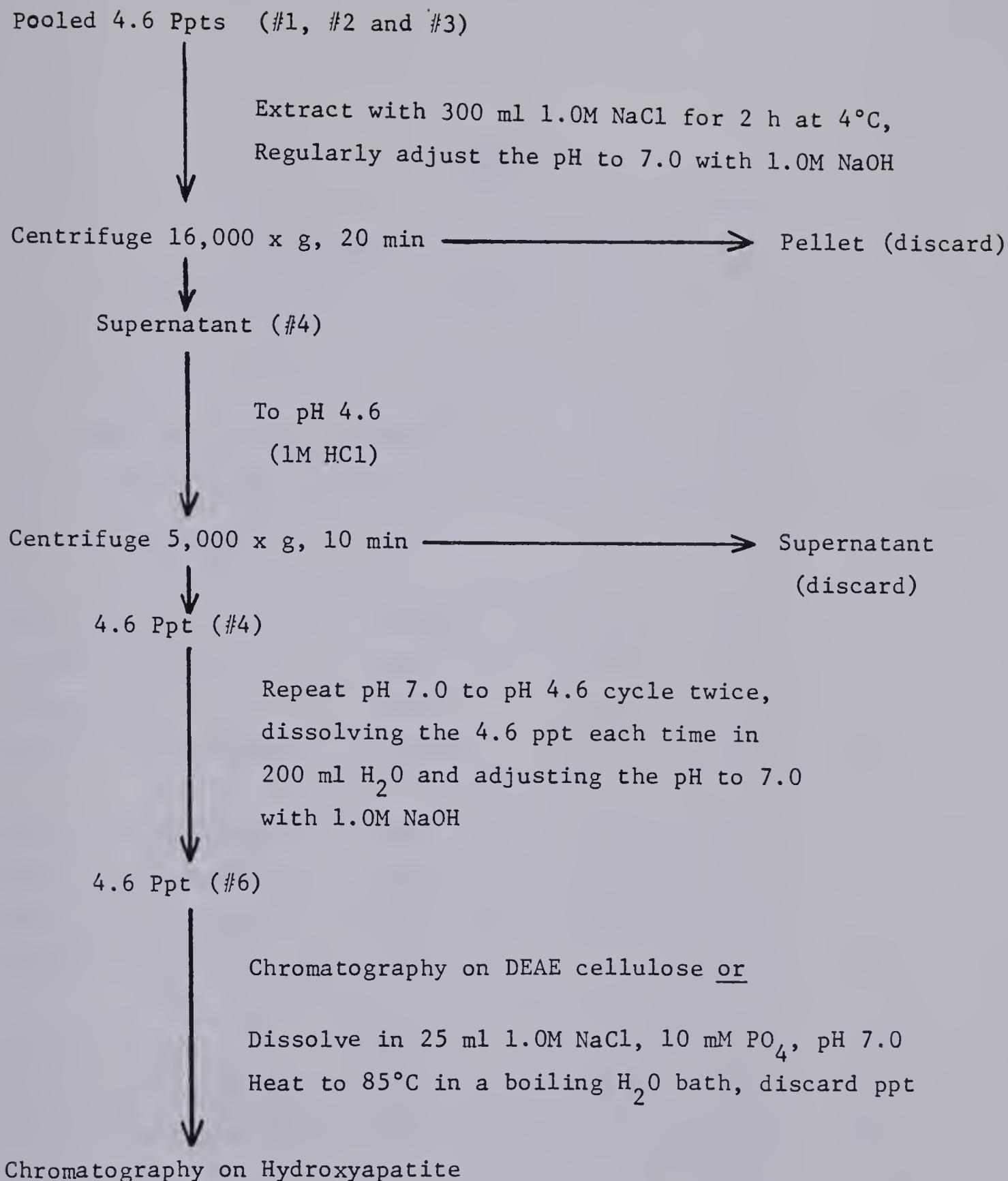


Fig. 13. Flow chart of the purification of platelet Tm by method 1. Yields and amounts at each step are given in Table IV. Gels for various steps are shown in Fig. 14.

TABLE IV

 Purification of Tm from Platelets by
 Method 1

Fraction	Total ^a Protein (mg)	Tm ^b (%)	Tm (mg)	Yield (%)
Initial homogenate	4700	1.2	56	100
First extract	3000	1.0	30	
Second extract	650	1.3	8	
Third extract	220	2.9	7	
Total of the extracts	3870		45	80
pH 4.6 precipitate #1	780	3.3	26	
pH 4.6 precipitate #2	160	3.9	6	
pH 4.6 precipitate #3	90	7.3	7	
Pooled 4.6 precipitates	1060	3.8	39	69
Supernatant #4	440	7.6	33	60
pH 4.6 precipitate #4	270	10.8	29	52
pH 4.6 precipitate #6	160	17	27	48
Soluble after 85°C treatment	45	45	20	36
DEAE chromatography	25	60	15	27
Hydroxyapatite	11	>95	11	20

^a Determined by amino acid analysis or the method of Bradford (1976).

^b Determined by densitometry of stained gels.



Fig. 14. SDS polyacrylamide gradient gels showing various steps of the purification of platelet Tm by method 1, (a) standards; bovine serum albumin, actin, platelet Tm, Tm-I, (b) 4.6 ppt #1, (c) 4.6 ppt #2, (d) supernatant #4, (e) discarded pellet at extraction of supernatant #4, (f) standards as in (a), (g) 4.6 ppt #4, (h) insoluble at pH 7.0 after 4.6 ppt #4, (i) 4.6 ppt #6, (j) ppt from the heat denaturation step, (k) supernatant from the heat denaturation step

various stages. Total protein was determined by either the dye binding method of Bradford (1976) or by amino acid analysis, while the percentage of Tm present was obtained by scanning gels and measuring, by planimeter, the total protein area and the area of the Tm peak. Because of the difficulties associated with this method yields should only be considered as a rough guide.

The platelets used for the purification of Tm had only been washed once and so contained some protein derived from red blood cells and plasma. The initial homogenate contained 75 - 80% platelet protein so that close to 1.2% of the starting material was Tm.

The platelets were extracted three times, twice with buffer containing 0.1M NaCl and once in 1.0M NaCl. During each extraction the platelets were homogenized for 15 seconds in a Waring blender. The basic idea at this stage was to solubilize as much of the platelet protein as possible and then purify the Tm by means of relatively selective and quantitative isoelectric precipitations at pH 4.6. To avoid losing platelet Tm the isoelectric precipitations should not be performed at protein concentrations of less than 1 mg/ml.

After stirring at pH 7.0 for 2 to 3 hours much of the protein which co-precipitated with Tm at pH 4.6 would not redissolve again. This insoluble material, usually brownish in colour, was difficult to centrifuge down unless high speeds were used.

The purification procedure can be suspended at any convenient stage of the 4.6 precipitations by means of lyophilization. In fact this process often assists the purification of platelet Tm by denaturing other proteins, thus rendering them insoluble at pH 7.0.

By the time of the final pH 4.6 precipitation (#6) Tm comprised about 15% of the total protein. Two methods were used to purify the Tm further. The simplest procedure was to dissolve the protein in 1.0M NaCl and place it in a boiling water bath until the temperature of the solution reached 85°C. Protein precipitated by the heat was then removed by centrifugation and the Tm recovered by lowering the pH of the supernatant to 4.6. This method is quick, removes a great deal of contaminating protein and probably denatures most proteases that may be present. Its disadvantage is that it is a fairly harsh treatment and may alter the properties of the Tm. There is some evidence that, upon heating, skeletal Tm gradually loses its ability to regulate an actomyosin ATPase system (Cummins & Perry, 1973).

For this reason an alternative procedure, involving chromatography upon DEAE cellulose, was developed to prepare platelet Tm for biological studies. A typical column profile (Fig. 15) displayed a number of peaks, the first of which was brownish in colour, ran poorly on gels and appeared to contain little protein. Platelet Tm eluted later at a KCl concentration close to 0.3M. This is very similar to the salt concentration at which skeletal Tm elutes from DEAE cellulose.

Final purification of the Tm was performed, in all cases, on hydroxyapatite, following basically the procedure of Eisenberg and Kielly (1974). Platelet Tm, like skeletal, eluted at the very end of the PO₄ gradient (0.2M) and was the last peak off of the column (Fig 16). At this stage the protein displayed a single band on SDS gels and was judged to be at least 95% pure.

Fig. 15a. DEAE cellulose chromatography of platelet Tm, previously purified by isoelectric precipitations. Protein (160 mg), dialyzed overnight against 0.15M KCl, 10 mM PO₄, 2 mM DTT, pH 7.0, was centrifuged to remove insoluble material and applied to a DEAE cellulose (Whatman) column (1.5 x 15 cm) equilibrated against the same buffer and eluted at 20 ml/h with a linear gradient (400 ml total volume) to 0.5M KCl.

Fig. 15b. Fractions from the DEAE column electrophoresed on an SDS urea gel. (a) material insoluble in the column starting buffer. (b) material applied to the column. (c to j) material corresponding to the pooled fractions with the same letters in Fig. 15a.

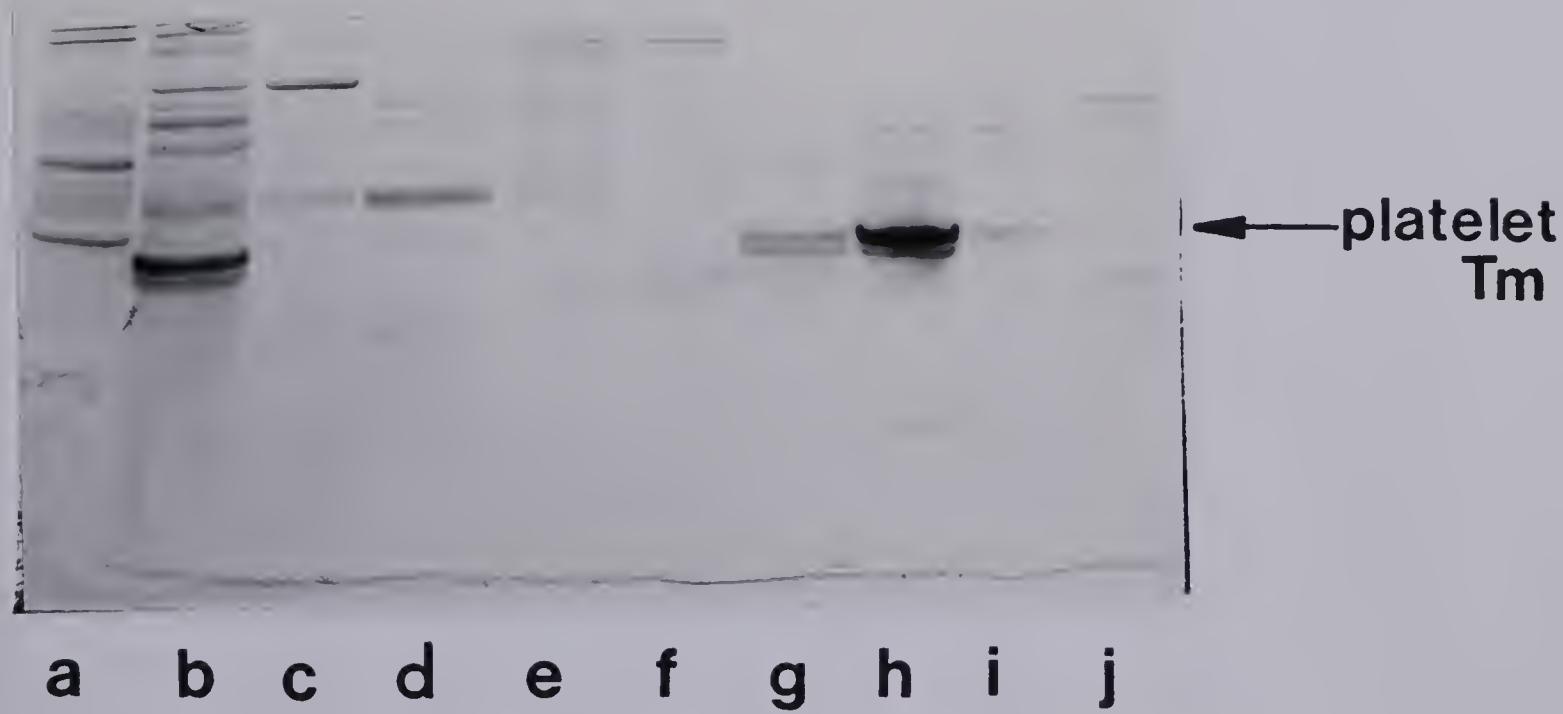
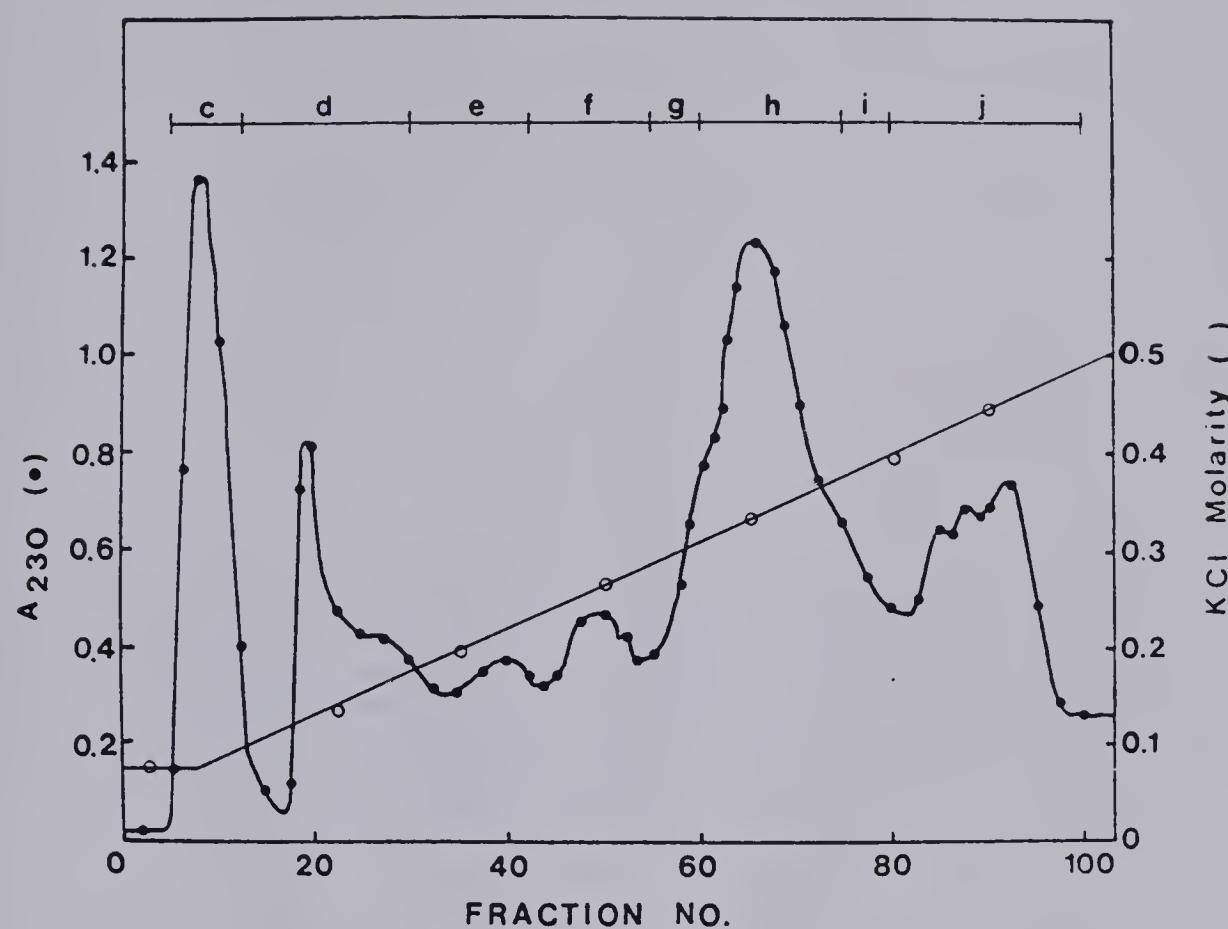
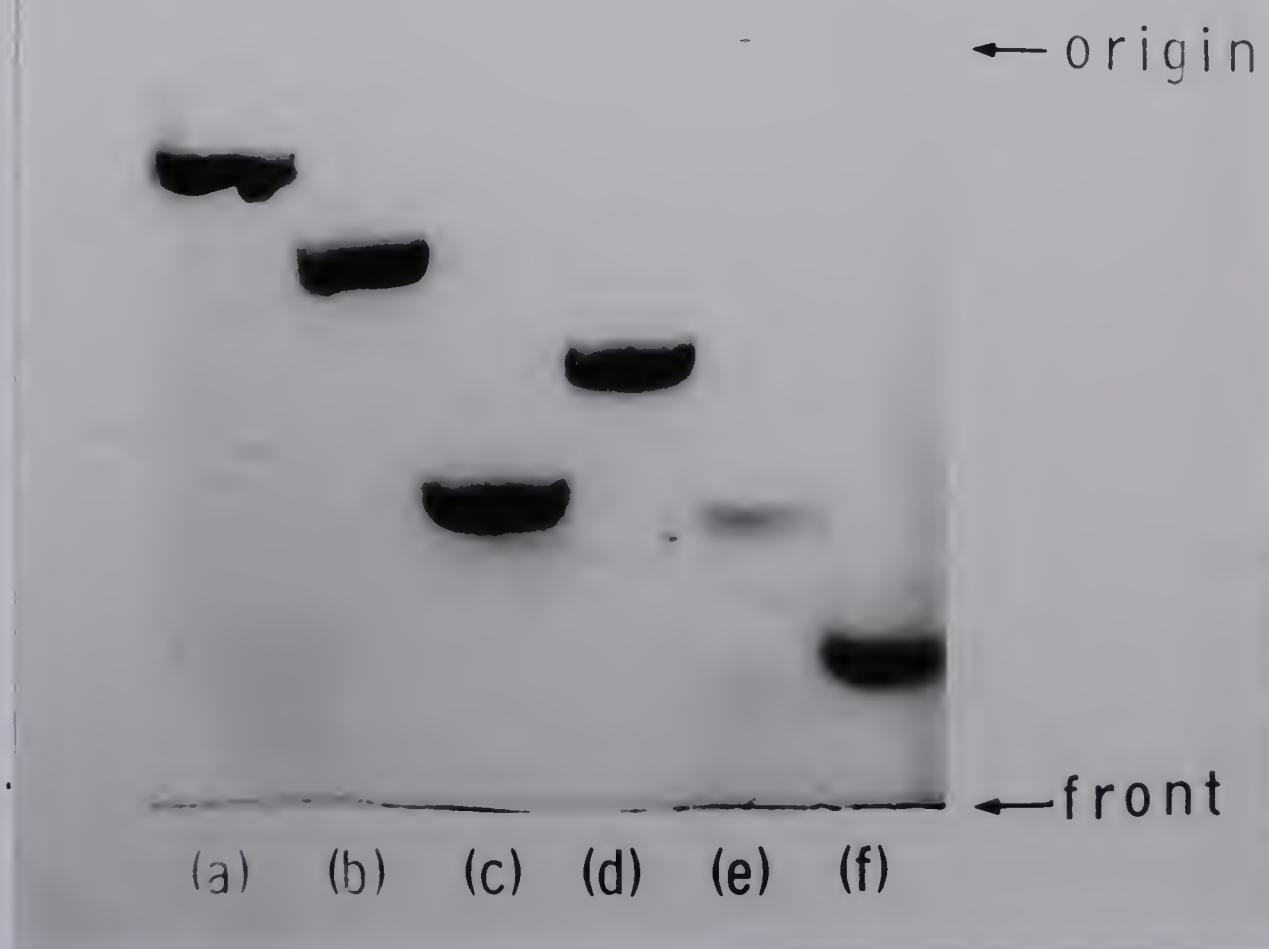
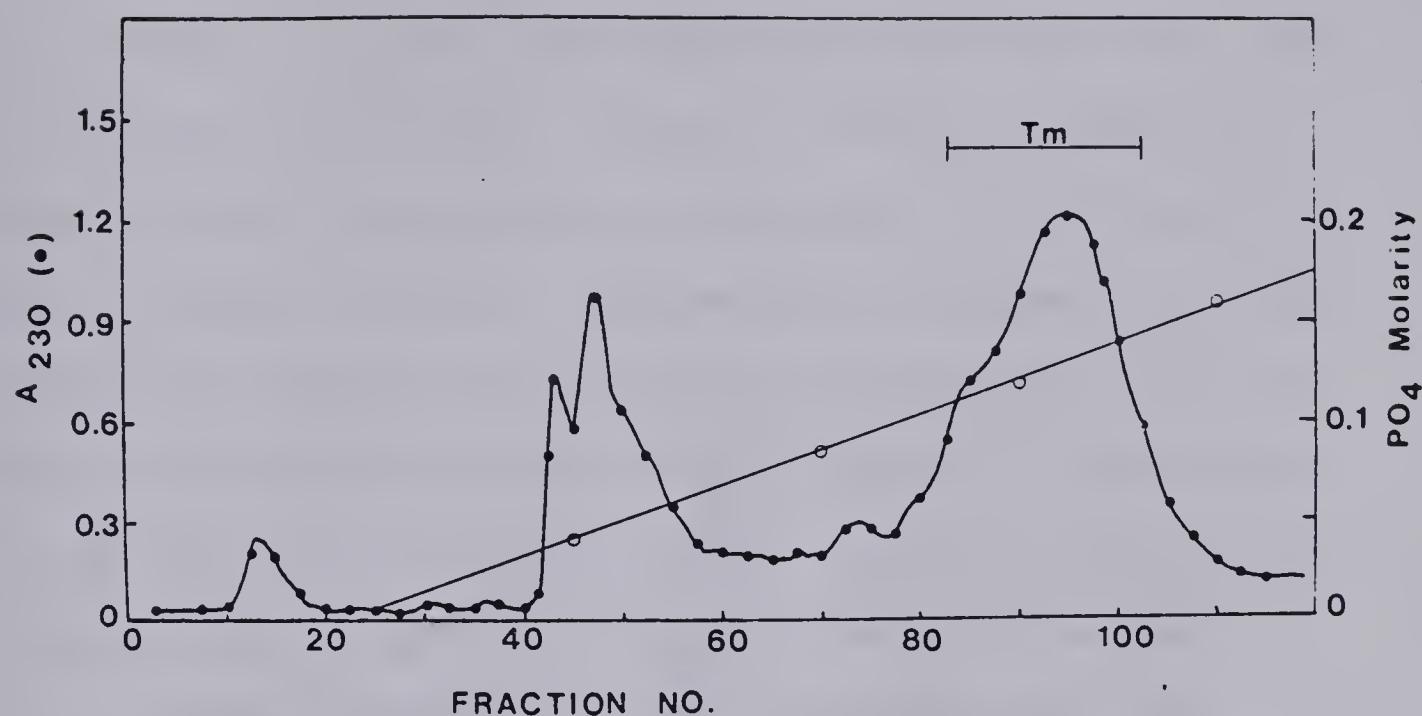


Fig. 16a. Hydroxyapatite chromatography of platelet Tm, purified in the previous step by chromatography on DEAE cellulose. Protein (50 mg) in 10 ml of 1.0M KCl, 2 mM DTT, 1 mM PO₄, pH 7.0 was applied to a hydroxyapatite (Bio-Rad DNA grade) column (2.0 x 20 cm) equilibrated against the same buffer. The protein was eluted at 20 ml/h with a 300 ml gradient of PO₄ to 0.2M. Each fraction contained 3.3 ml.

Fig. 16b. SDS polyacrylamide gels (Weber & Osborn, 1969) of (a) phosphorylase, (b) bovine serum albumin, (c) platelet Tm pooled as indicated from the hydroxyapatite column in Fig. 16a, (d) ovalbumin (e) chymotrypsinogen, (f) ribonuclease.



4. Purification Method 2

Method 1 was developed as a means of isolating platelet T_m without recourse to harsh, denaturing conditions which could affect the structure of the protein. However, a number of studies, including following the temperature denaturation of the protein by means of circular dichroism, indicated that the protein could readily renature. Method 2 was designed to take advantage of the stability of the platelet T_m's coiled-coil structure in order to purify it in less time and in greater yields. Details of the procedure are given in Fig. 17 and Table (V), while SDS gels of material obtained at various stages of the purification are shown in Fig. 18.

The major change from method 1 is the buffer used in the initial extraction, which contains 25 mM HCl and has a pH of 2.

Under these conditions the coiled-coil of T_m is stable (Lowey, 1965) but many of the other proteins would appear to be denatured. They are extracted, but precipitate out at pH 4.4 and do not redissolve again at pH 7.8. This provides a greater than ten times purification of the platelet T_m in a single step. The result is that by the second of the pH 4.4 precipitations the platelet T_m is almost 60% pure.

The next step, heating in a boiling water bath, removes most of the remaining impurities. Hydroxyapatite chromatography provides the final purification (Fig. 19). The final product, judged to be greater than 95% pure, is shown in the inset to Fig. 19. The darkest band above the major band of both platelet and skeletal T_m represents a dimer of two T_m chains linked by a disulfide bond

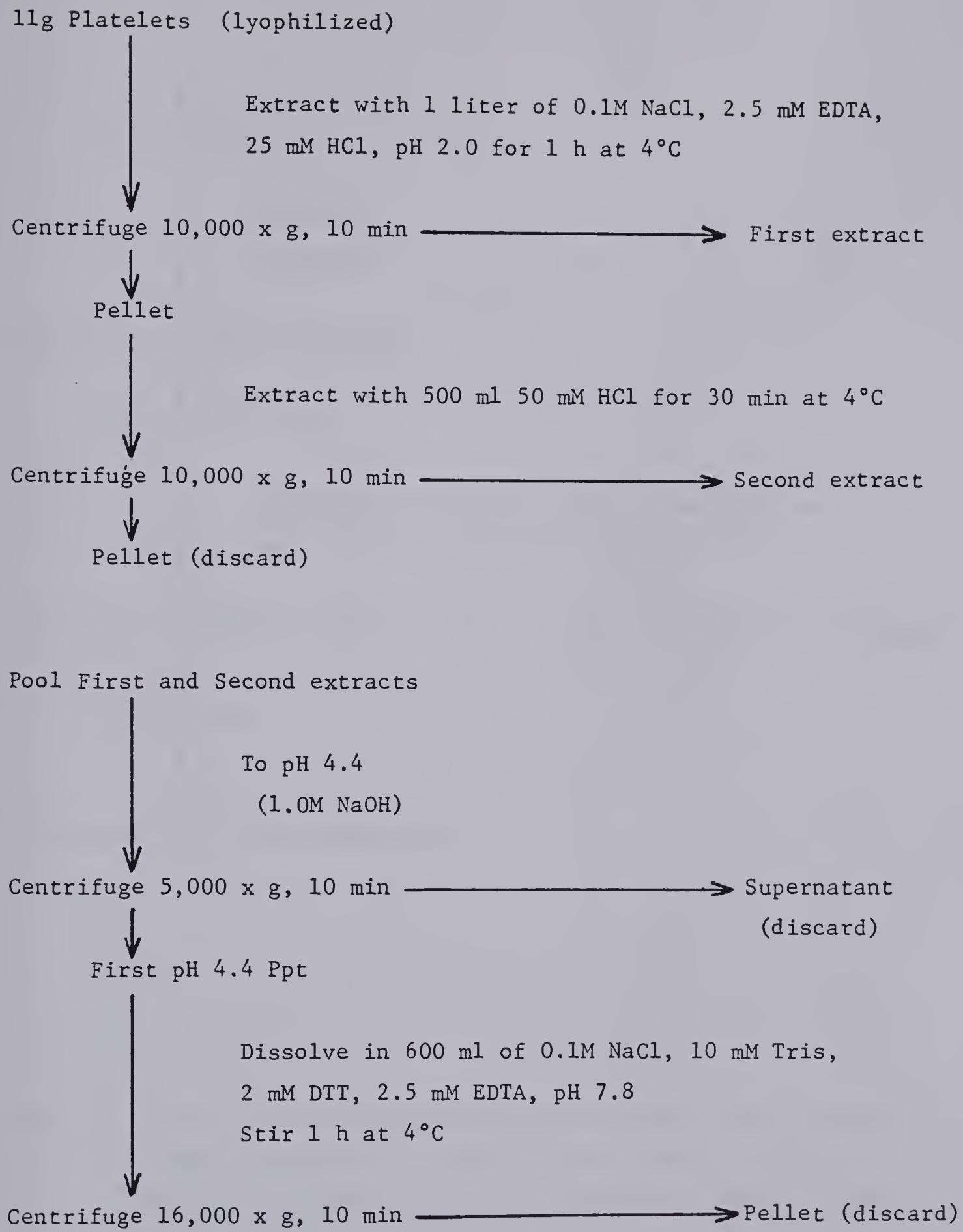


Fig. 17. (continued next page)

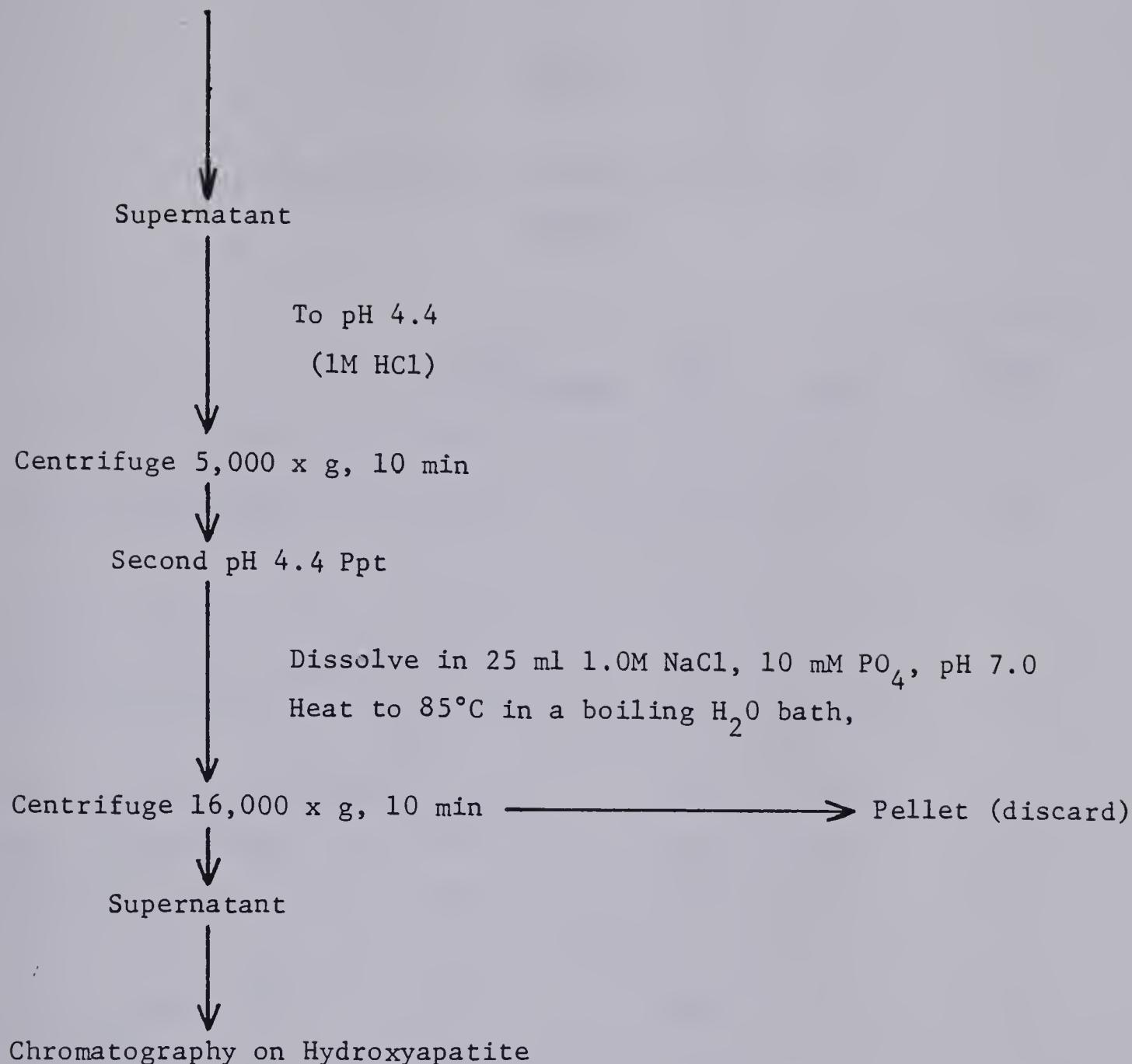


Fig. 17. Flow chart of the purification of platelet Tm by method 2. Yields and amounts at each step are given in Table V. SDS gels of samples at various stages are shown in Fig. 18.

TABLE V

 Purification of Tm from Platelets by
 Method 2

Fraction	Total ^a Protein (mg)	Tm ^b (%)	Tm (mg)	Yield (%)
Initial homogenate	6000	1.2	72	100
First extract	2840	1.8	51	
Second extract	890	1.6	14	
Pooled extracts	3730	1.8	65	90
First pH 4.4 precipitate	2170	2.4	52	72
pH 7.8 Supernatant	105	33	35	49
Second pH 4.4 precipitate	49	59	29	40
Soluble after 85°C treatment	30	90	25	35
Hydroxyapatite chromatography	22	>95	22	30

^a Determined by amino acid analysis or the method of Bradford (1976).

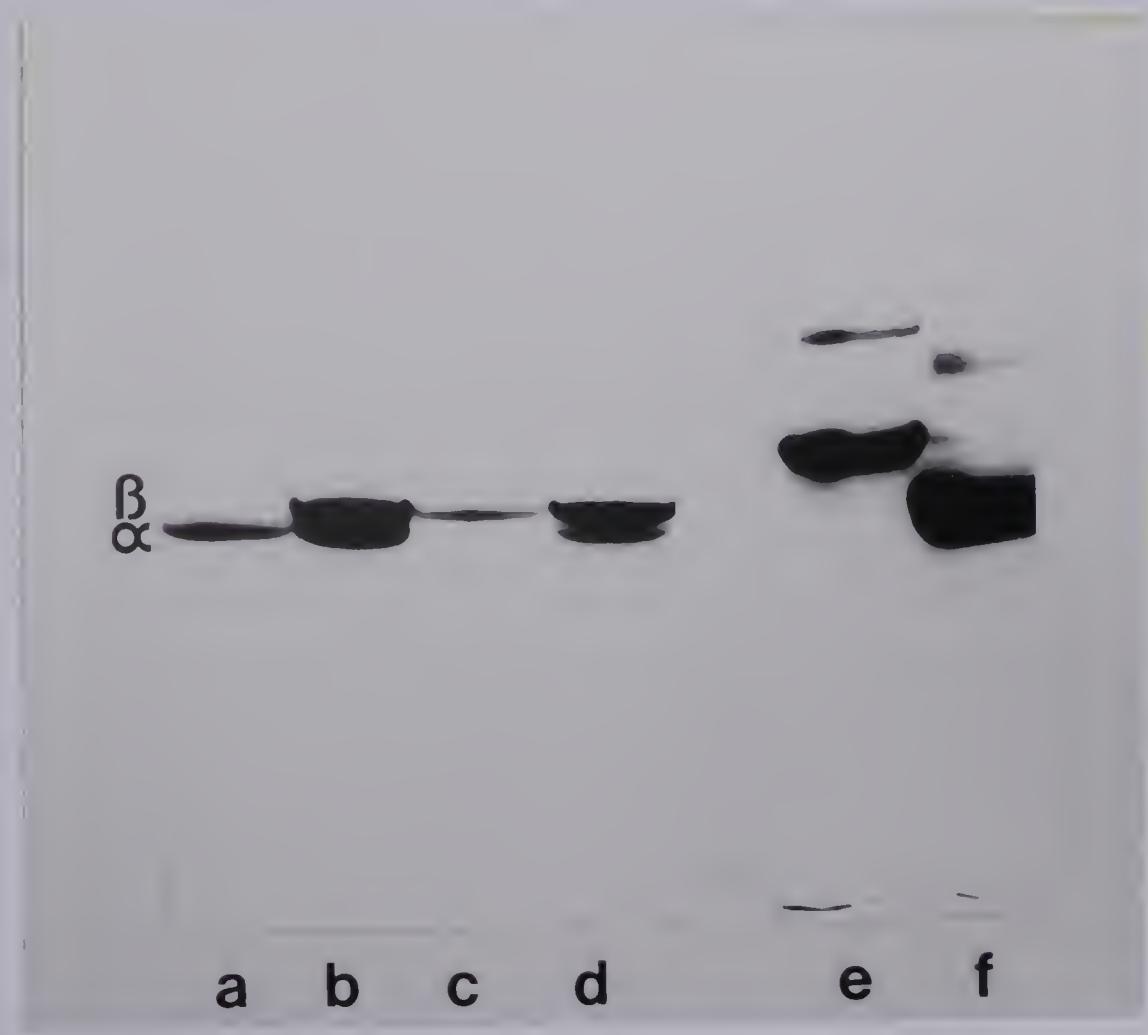
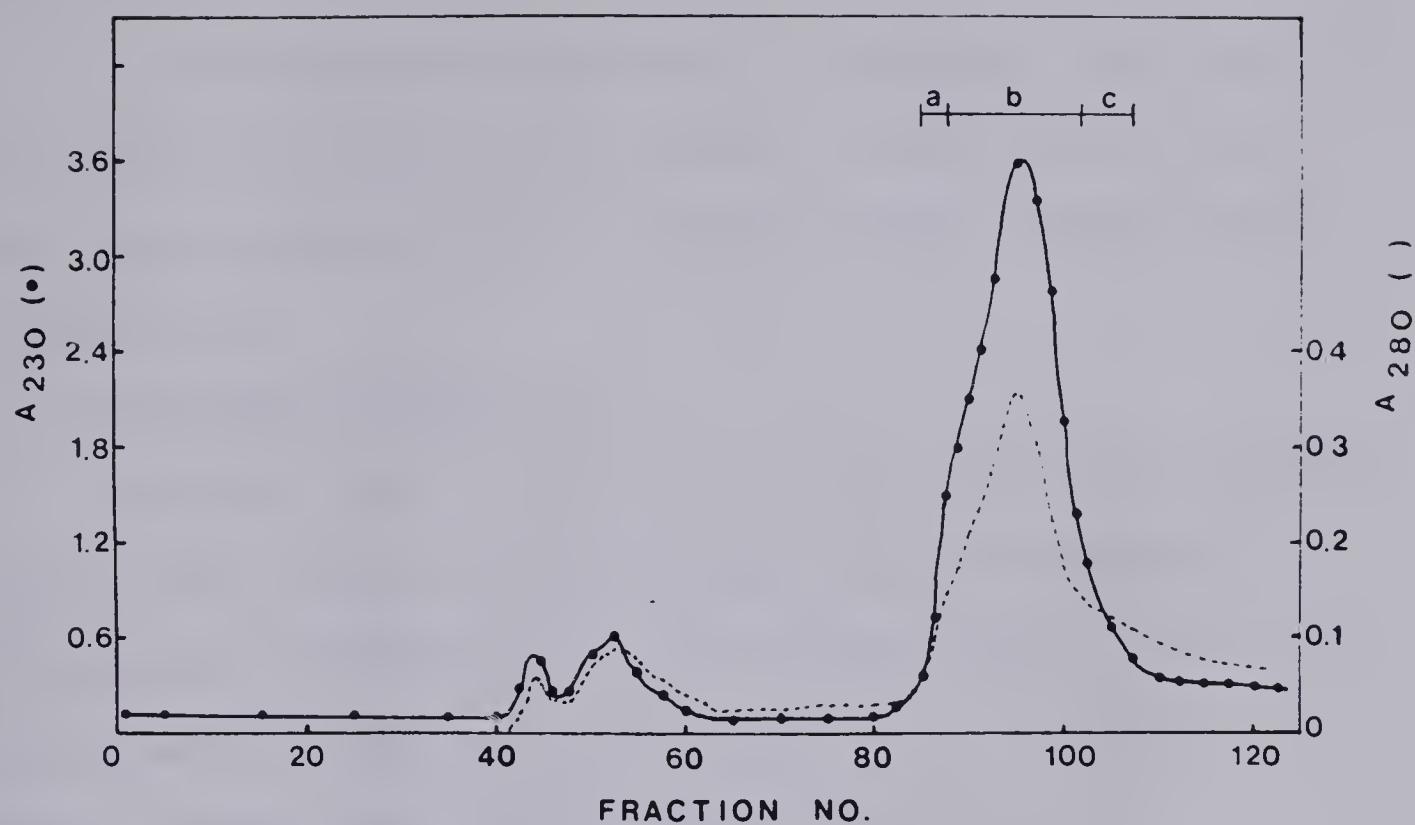
^b Determined by densitometry of stained gels.



Fig. 18. SDS polyacrylamide gradient gel showing various steps of the purification of platelet Tm by method 2, (a) whole platelets, (b) first extract, (c) second extract, (d) unextracted residue, (e) second 4.4 supernatant, (f) first pH 4.4 supernatant, (g) standards; bovine serum albumin, actin, platelet Tm, Tn-I, (h) first pH 4.4 ppt, (i) second pH 4.4 ppt, (j) insoluble at pH 7.8

Fig. 19a. Hydroxyapatite chromatography of platelet Tm purified by method 2. The column was run as described in the legend to Fig. 16a, with the exception that the sample size was 110 mg and the gradient volume was 600 ml. Each fraction contained 4 ml and the gradient was started at tube 10. The solid line indicates A_{230} while the broken line is A_{280} .

Fig. 19b. Fractions from the hydroxyapatite column electrophoresed on SDS urea (a,b,c) or SDS (e,f) gels. (a,b,c) correspond to the pooled fractions of the same letter in Fig. 19a. There is partial separation of the two forms of platelet Tm by the hydroxyapatite column. (d) material in slot b rerun at a lower loading. (e) skeletal α Tm. (f) platelet Tm greatly overloaded; this is the same sample as run in slots b and d.



(Fig. 19e,f).

As discussed more fully later, no significant differences in the properties of platelet Tm prepared by method 1 and 2 have been found, although protein prepared by method 2 appears to be slightly purer.

5. Proteolysis in Platelets

Platelets contain a number of proteases (see for ex. Legrand et al., 1977), so that it must be asked whether the smaller size of platelet Tm, in comparison with the skeletal molecule, is a result of proteolysis occurring during purification. Two pieces of evidence, however, make such a possibility unlikely.

Firstly, preparations of whole platelets, when subjected to SDS gel electrophoresis, show a distinct protein band in the position expected for the smaller Tm (Fig. 11). The mobility of this band decreases on SDS urea gels indicating its Tm like nature. Therefore the smaller Tm is present in platelets before the purification commences. If the platelet protein is produced from a larger Tm precursor, then the limited proteolytic event has already occurred in the intact platelet.

Secondly, if a larger form of Tm is present in platelets there is no reason to expect that it would not co-purify with the smaller platelet Tm. Although platelet Tm was isolated in large amounts during this study, and by various methods, no evidence was ever found for the existance of a form of Tm with a higher M.Wt.

D. TWO FORMS OF PLATELET TM

Platelet Tm electrophoreses as a single band on SDS gels

prepared according to the method of Weber and Osborn (1969). If, however, the protein is run of SDS gels containing 6M urea (Sender, 1971) or on gels prepared after the method of Laemmli (1970), two bands are observed (Figs. 19 & 20). In conformity to the nomenclature adopted for muscle Tm the faster moving component is designated as α , and the slower as β , platelet Tm.

On SDS urea gels, but not on Laemmli gels, two higher M.Wt. bands can sometimes be seen above β platelet Tm (Fig. 21). The topmost band is a dimer of platelet Tm subunits, held together by a disulfide bond, and is greatly increased on SDS gels when reducing agents are omitted. The formation of such a dimer appears to occur more readily in SDS urea. The presence of the second minor component, running between β platelet Tm and the dimer, is more difficult to explain. It is usually a diffuse, spread out band which does not seem to be caused by a single type of protein. A possible explanation is that it results from the formation of an intra-chain disulfide bond within one of the subunits of platelet Tm (as demonstrated in Chapter IV each platelet Tm chain contains two cysteines). The change in conformation produced by introduction of the disulfide bond could alter the mobility of the subunit (Weber et al., 1972)

During the purification of platelet Tm it was noticed that in certain instances a partial separation of the α and β forms of this protein occurred. Chromatography on either hydroxyapatite or DEAE led to the elution of the platelet protein as a single peak; however, the leading edge of the peak was always enriched in the α component, while the trailing edge contained predominately the β

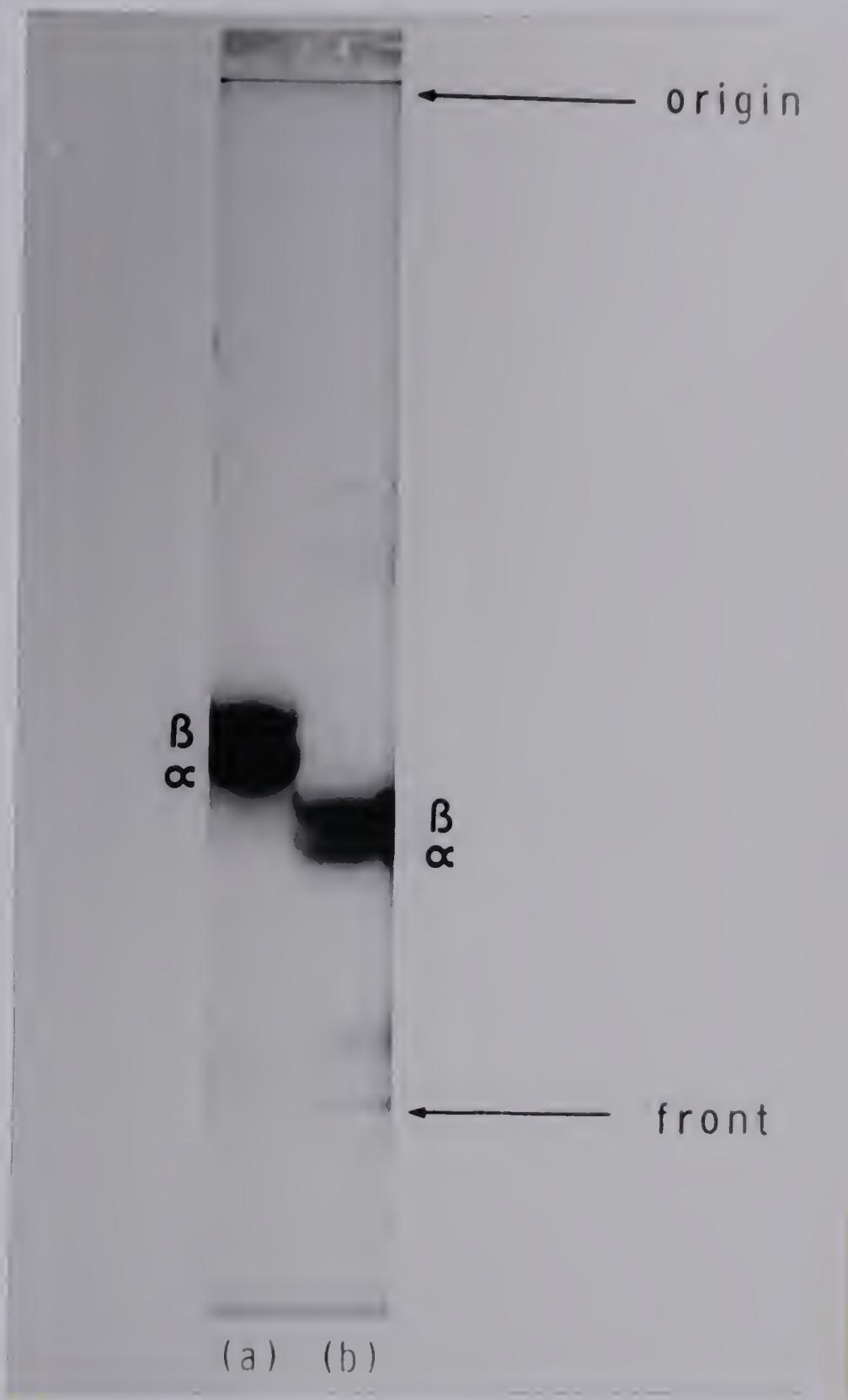


Fig. 20. SDS gel (Laemmli, 1970) of (a) skeletal α and β Tm and (b) platelet Tm, showing the two forms of this protein which have been designated as α and β .

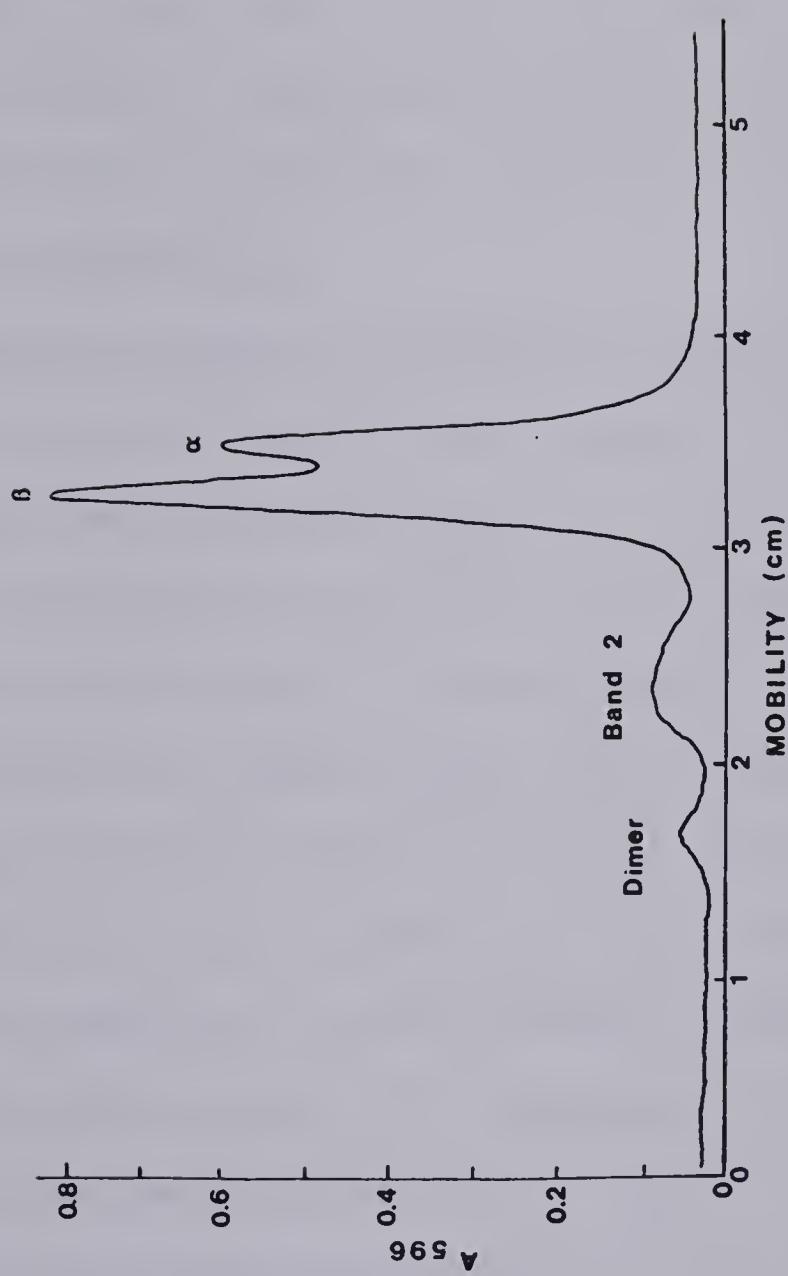


Fig. 21. Platelet Tm, prepared by method 2, electrophoresed on an SDS gel in the presence of 6M urea, and scanned at 596 nm. The platelet Tm, which gave a single band on SDS gels, split into two major bands, α and β , as well as two minor bands, a dimer of two Tm chains and an unknown Band 2, when run on this gel system.

component. Examples of such separations can be seen for the hydroxyapatite column in Fig.19 and the DEAE cellulose column in Fig. 15. It was also found that ammonium sulfate precipitations could partially separate the α and β components.

An analysis of twelve different preparations of platelet Tm (ten prepared by method 1) revealed that the amount of Tm present as the α form varied from 25 to 40%, the average value being 33%. Part of this variation could result from the partial loss of one form or the other during purification, especially on pooling fractions from column chromatography.

Platelet Tm prepared by method 2 was pure enough to be analyzed for its α and β content before passage through any column. These samples (material which precipitated during the second pH 4.4 treatment and material soluble after the 85°C step) yielded values of α varying from 29 to 33%. These figures should be close to the in vivo values for it seems unlikely that the purification up to these stages would have selected for either of the two forms.

Attempts were made to separate the two chain types by the method developed for separating α and β skeletal Tm (Cummins & Perry, 1973). This involves chromatography on a carboxymethyl cellulose column at pH 4.0 in the presence of 8M urea to denature the protein. The Tm is usually carboxymethylated to ensure that disulfide bonds do not link chains together. This procedure could not be used with the platelet protein because after carboxymethylation the α and β forms could no longer be separated on SDS urea gels; they electrophoresed together as a single band slightly above the position

expected for the β form.

An alternative method to prevent disulfide bridge formation is to run the carboxymethyl cellulose column in the presence of reducing agent (5 mM DTT was used). Platelet Tm subjected to this procedure eluted as a single peak with no indication, judging by samples taken across the peak, of any separation of the α and β forms.

E. DISCUSSION

Tm is a difficult protein to characterize biologically because alone it has no activity; it can only be assayed when interacting in a complex manner with troponin, actin and myosin. These assays cannot be performed with Tm that is in an impure state, so a purification scheme cannot then be devised to isolate a protein on the basis of its ability to function in a Tm like manner. What can be accomplished is to isolate a protein which displays the same chemical and physical properties as Tm. Since Tm possesses a number of distinctive properties which set it apart from the majority of proteins, a purification scheme based on these properties should isolate a molecule sharing many of Tm's structural characteristics.

The evidence from the purification of the platelet protein that indicates its similarity to skeletal Tm is:

- 1) its stability at high temperatures,
- 2) its stability at extremes of pH,
- 3) its insolubility at or close to the isoelectric point of muscle Tm (pH 4.6),
- 4) its solubility in H_2O at neutral pH,

5) its elution from hydroxyapatite and DEAE cellulose columns near the known elution position of skeletal T_m,

6) its altered mobility on SDS urea gels as compared to SDS gels.

Such results certainly do not prove that the protein is a T_m. For example, proteins recently isolated from certain bacteria display similar physico-chemical similarities to T_m, yet appear to be completely unrelated functionally (Hosein et al., 1979). For convenience the platelet protein has been referred to as platelet T_m, although it is understood that in order to unequivocally establish its identity further characterization is required.

The two methods discussed in this chapter are suitable for purifying large amounts of platelet T_m to a high degree of purity. Method 1 has the advantage that the platelet T_m is not subjected to denaturing conditions while method 2 produces better yields, is faster and produces a final product which is slightly purer. Method 2, especially, should be suitable for the purification of Tms from a wide variety of non-muscle sources.

Method 1 and method 2 recover the platelet T_m in yields of 20 and 30% respectively, based on a T_m content in platelets of 1.6%. Previously published purification methods (Cohen & Cohen, 1972; Fine et al., 1973) have not reported yields since the amount of T_m originally present in their starting materials was not determined.

Actin was found to constitute 16% of the total platelet protein, in reasonable agreement with earlier values which ranged from 10 to 25% (Stossel, 1978; Gordon et al., 1977). There would thus be

13 or 14 molecules of actin per molecule of Tm in platelets. It has been estimated that 50 - 75% of actin, in unactivated platelets, is held in the monomeric state by profilin (Markey et al., 1978; Blikstad et al., 1978). Assuming that one platelet Tm binds to six actins, this means that there is likely enough Tm present in platelets to cover all the available F-actin filaments.

SDS urea gels or Laemmli gels revealed that there were two different forms of platelet Tm (designated as α and β). Bretscher and Weber (1978) found that Tm isolated from bovine brain gave rise to two bands when run on these same gel systems. In both the brain and the platelet case the β component appears to be present in the largest amount, indicating that Tm molecules composed of two β chains ($\beta\beta$) must exist.

A number of procedures have been found which partially separate the α and β forms of platelet Tm, suggesting that they must differ in some of their properties. Under non-denaturing conditions a fraction containing a higher proportion of α than β chains can be obtained, indicating that $\alpha\alpha$ dimers must also occur naturally. Whether only these two distinct forms of platelet Tm are present or whether some percentage of the protein consists of mixed α and β subunits is not known.

Attempts to completely separate the two forms of platelet Tm by chromatography under denaturing conditions were not successful. However, because the two chain types appear to have definite, although slight, differences in their properties, the purification of each should be feasible.

By analogy to the situation with skeletal muscle α and β Tm, it is expected that the two forms of platelet Tm will differ only slightly in amino acid sequence and not in M.Wt. (Mak et al., 1979). Even with the complete sequences of α and β skeletal Tm known, it is not apparent why the two forms should be separated on SDS urea gels. It is not possible therefore to predict what differences between α and β platelet Tm are responsible for their varying mobilities on Laemmli and SDS urea gels.

CHAPTER IV
CHEMICAL AND PHYSICAL CHARACTERIZATION
OF PLATELET TM

The studies in this chapter were undertaken to further define the purity of the platelet protein, to establish its identity as a Tm, and to determine in what manner it resembled or differed from the skeletal protein.

Results of the physical and chemical techniques used, to be discussed in this chapter, leave little doubt as to the close relationship between the platelet and skeletal molecules.

A. MOLECULAR WEIGHT DETERMINATIONS

1. SDS Gel Electrophoresis

It is well established that electrophoresis on polyacrylamide gels in the presence of SDS separates proteins on the basis of their M.Wts. (Weber & Osborn, 1969). By plotting the mobility of standard polypeptides relative to the dye front against the log of their M.Wts. a straight line is obtained which can be used to estimate the M.Wts. of unknown proteins.

On 8% SDS gels platelet Tm had a relative mobility of 0.63, corresponding to a M.Wt. of 28,500, while cardiac Tm had a mobility of 0.58, equivalent to a M.Wt. of 32,500 (Fig. 22). Since SDS, in the presence of reducing agent, dissociates proteins into their constituent subunits, these values are for the individual chains of the Tm molecule. If samples of platelet Tm were run on SDS gels in the absence of reducing agent a band with a slower mobility (0.31) appeared. This band, M.Wt. 67,500, most likely results from the

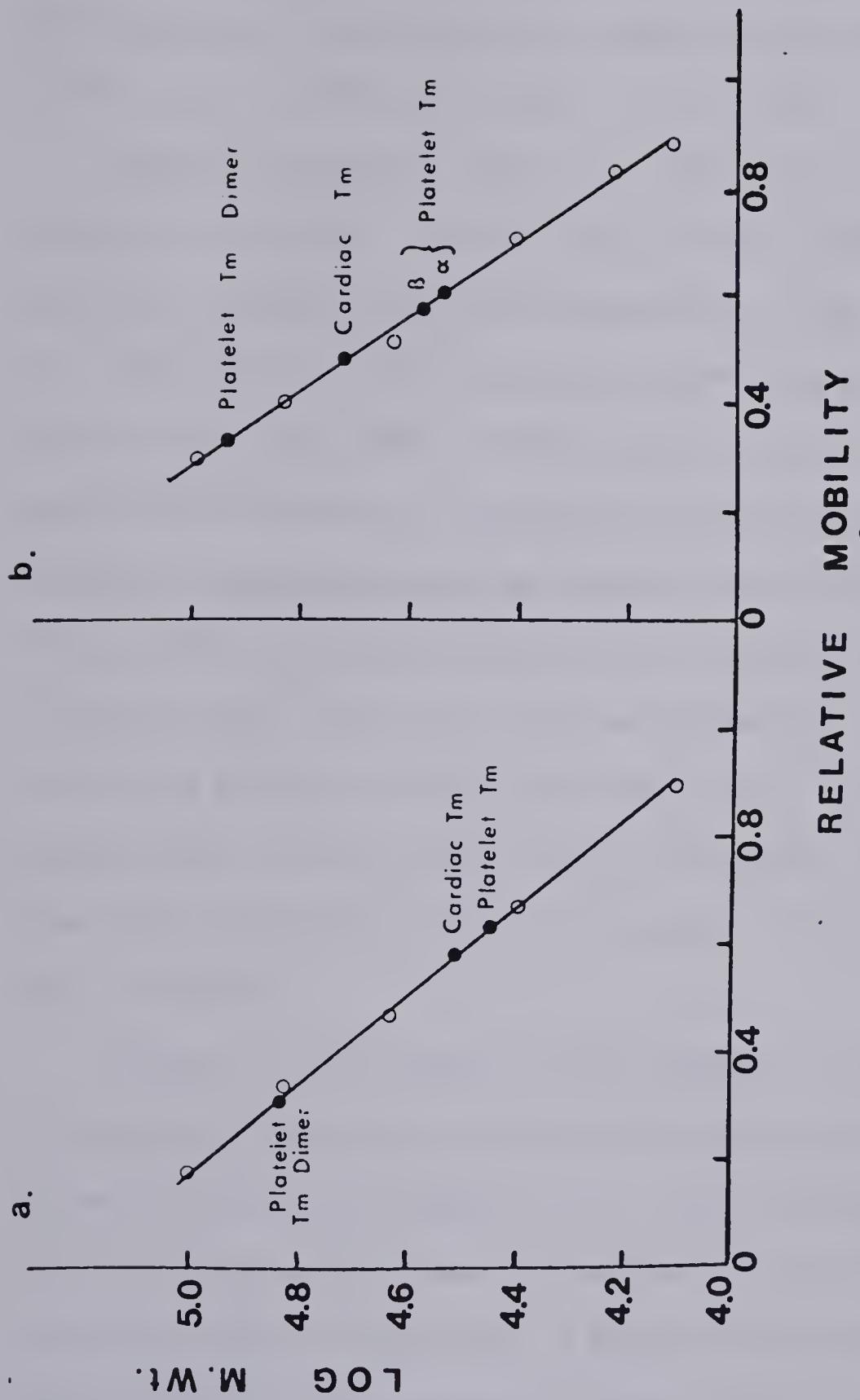


Fig. 22. Determination of the M.Wt. of platelet T_m by SDS gel electrophoresis. Samples were run on either, a) 8% SDS gels or, b) 8% SDS gels containing 6M urea. For each protein the log of its M.Wt. is plotted vs its mobility relative to the dye front. The standard proteins (o) and their M.Wts. are, from top to bottom, phosphorylase (100,000), bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen (25,700), myoglobin (16,900) (in b. only), ribonuclease (12,700) (Weber et al., 1972; Fasman, 1976)

formation of a disulfide bridge between the cysteines of two platelet T_m chains. The higher than expected M.Wt. of this dimer may perhaps be attributed to a loss of conformational freedom, caused by the introduction of these crosslinks, which in turn leads to a reduction in the amount of SDS bound (Weber et al., 1972).

Skeletal muscle T_m, when run on SDS gels containing 6M urea splits into two bands, termed α and β , both of which represent a different, although highly homologous form of the T_m subunit (Mak et al., 1979). The two chain types run on SDS urea gels with an anomalously high M.Wt., that is their mobility decreases with respect to those of other proteins. When run on SDS urea gels platelet T_m exhibits the same phenomena as skeletal T_m; it splits into two bands both of which display an apparent M.Wt. greater than that observed on SDS gels (Fig. 22b). The M.Wt. determined for cardiac T_m by means of SDS urea gels was 53,000, while the α and β components of platelet T_m gave M.Wts. of 35,000 and 38,000 respectively. The platelet T_m dimer also electrophoresed with a decreased mobility, to yield a M.Wt. of 86,000.

Skeletal T_m is almost the only protein, of the hundreds present in a myogenic cell line, to appreciably alter its mobility between SDS and SDS urea gels (Carmon et al., 1978) The reason it does so is not understood; perhaps some unique feature of its amino acid sequence causes the amount of SDS bound to be lowered below the standard value of 1.4g/g in the presence of urea. The fact that the platelet protein behaves in the same manner is evidence for an amino acid sequence, or at least a conformational, homology to the skeletal molecule.

The most accurate estimation of the M.Wt. of cardiac Tm was on SDS gels, where the value obtained was just slightly below that calculated from the sequence. Assuming the same holds for the platelet protein then its M.Wt. should be close to 28,500.

2. Ultracentrifugation

If the platelet molecule has a coiled coil structure composed of two chains similar to that of Tm, it should have a M.Wt. of about 57,000 under non-denaturing conditions. The M.Wt. under benign conditions was measured by the method of sedimentation equilibrium in the ultracentrifuge. A plot of the \ln of the concentration vs r^2 gave a straight line, the slope of which could be used to calculate a M.Wt. for the platelet protein of 63,800 (Fig. 23). This result, although 12% higher than the value derived from SDS gels, clearly indicates that the platelet protein, in its native state, consists of a dimer of the 28,500 dalton subunit.

B. GEL FILTRATION

M.Wts. can be determined by the elution position of a protein from a gel filtration column. Data are analyzed by plotting log M.Wt. vs Kd, the fraction of the gel internal volume accessible to the protein (Mann & Fish, 1972). When the gel filtration column is run under non-denaturing conditions Kd will depend partly on the shape of the protein (on its Stokes radius), but reasonably accurate M.Wts. can still be obtained for globular proteins. If high ionic strength buffers are used the only major deviations from linearity will occur for very asymmetric proteins such as Tm and fibrinogen.

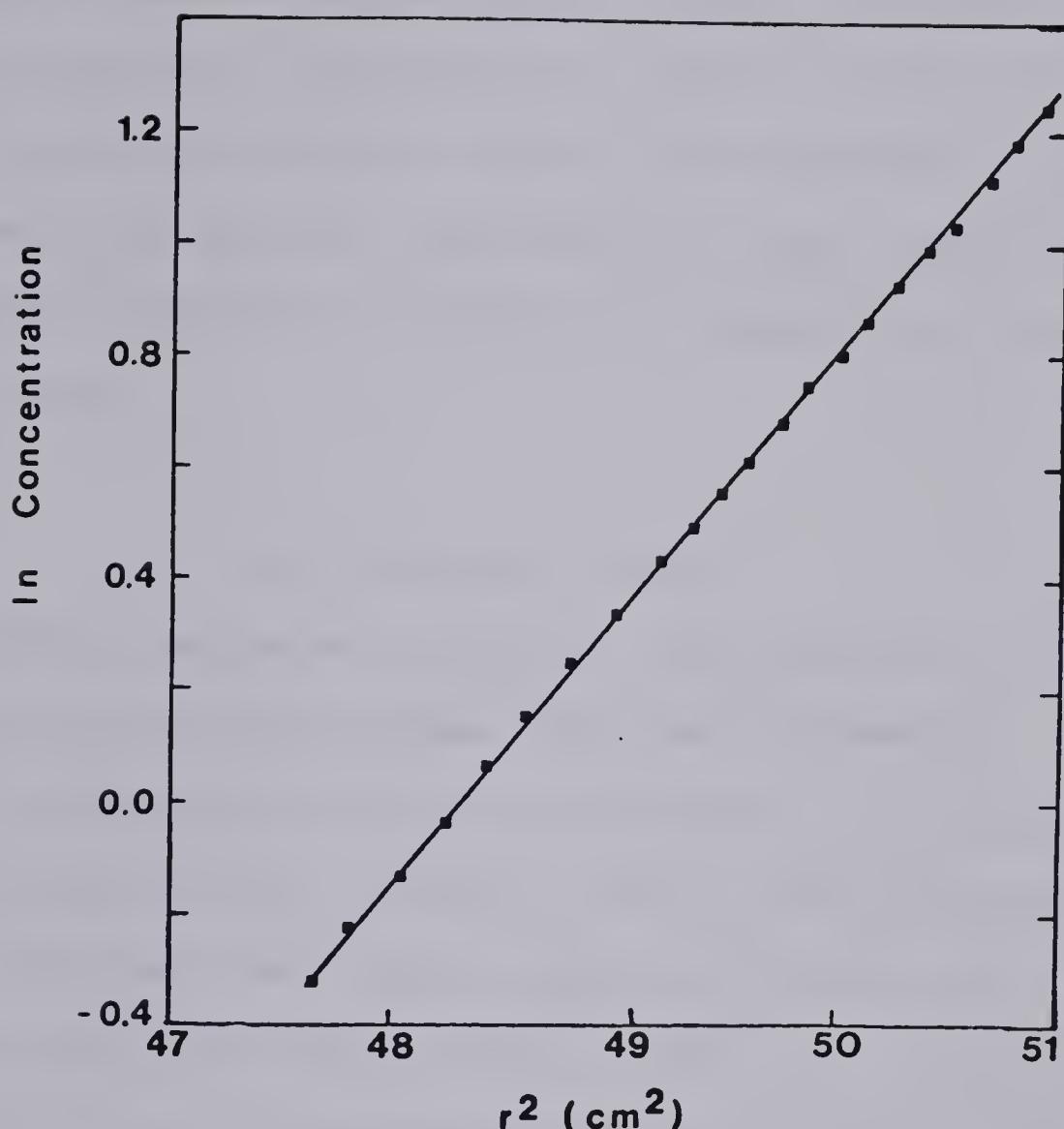


Fig. 23. A sedimentation equilibrium experiment performed in the ultracentrifuge with platelet Tm. The natural log of the concentration of platelet Tm is plotted against the square of the distance from the axis of rotation. The rotor speed was 13,000 rpm. \bar{v} was taken as 0.73 and ρ as 1.051. The original protein concentration, 2.03 mg/ml, was established in terms of fringes by a synthetic boundary experiment. The concentrations during the run were also determined by the Rayleigh interference fringe method. Platelet Tm was dialyzed overnight against 1.0M KCl, 1 mM DTT, 20 mM Tris, pH 7.5 before centrifugation.

A plot of K_d vs $\log M.Wt.$ for a BioGel A 1.5M column run in 1.0M NaCl reveals that skeletal Tm elutes at a position much earlier than would be expected on the basis of its M.Wt. The platelet protein elutes close to the position of skeletal Tm (Fig. 24). This result is only consistent with the M.Wt. of the platelet protein determined by sedimentation equilibrium if it has, like skeletal Tm, an asymmetric, rod-like conformation, and consequently a large Stokes radius.

C. AMINO ACID ANALYSES

Duplicate samples of platelet Tm, hydrolyzed for 24, 62 and 92 hours, were run on the Beckman 120c amino acid analyzer. The amount of each amino acid present was calculated by averaging the values from all six runs, except for isoleucine and valine where the 92 hour values were used, and for serine and threonine where the values were obtained by extrapolation to zero time. These values are shown in Table VI normalized to a M.Wt. of 28,500 in order to yield the number of amino acid residues per mole of platelet Tm chain. Values determined by summation from the known sequence of the major form of platelet Tm (Fig. 33) are also presented (Table VI).

The amino acid composition is very similar to that of skeletal Tm, and also to that of human platelet Tm isolated by Cohen and Cohen (1972). Particularly characteristic of Tm is the absence of proline, and the high concentrations of charged amino acids, especially glutamate. The only significant differences from skeletal α Tm appear to be the presence of twice as much glycine and only one

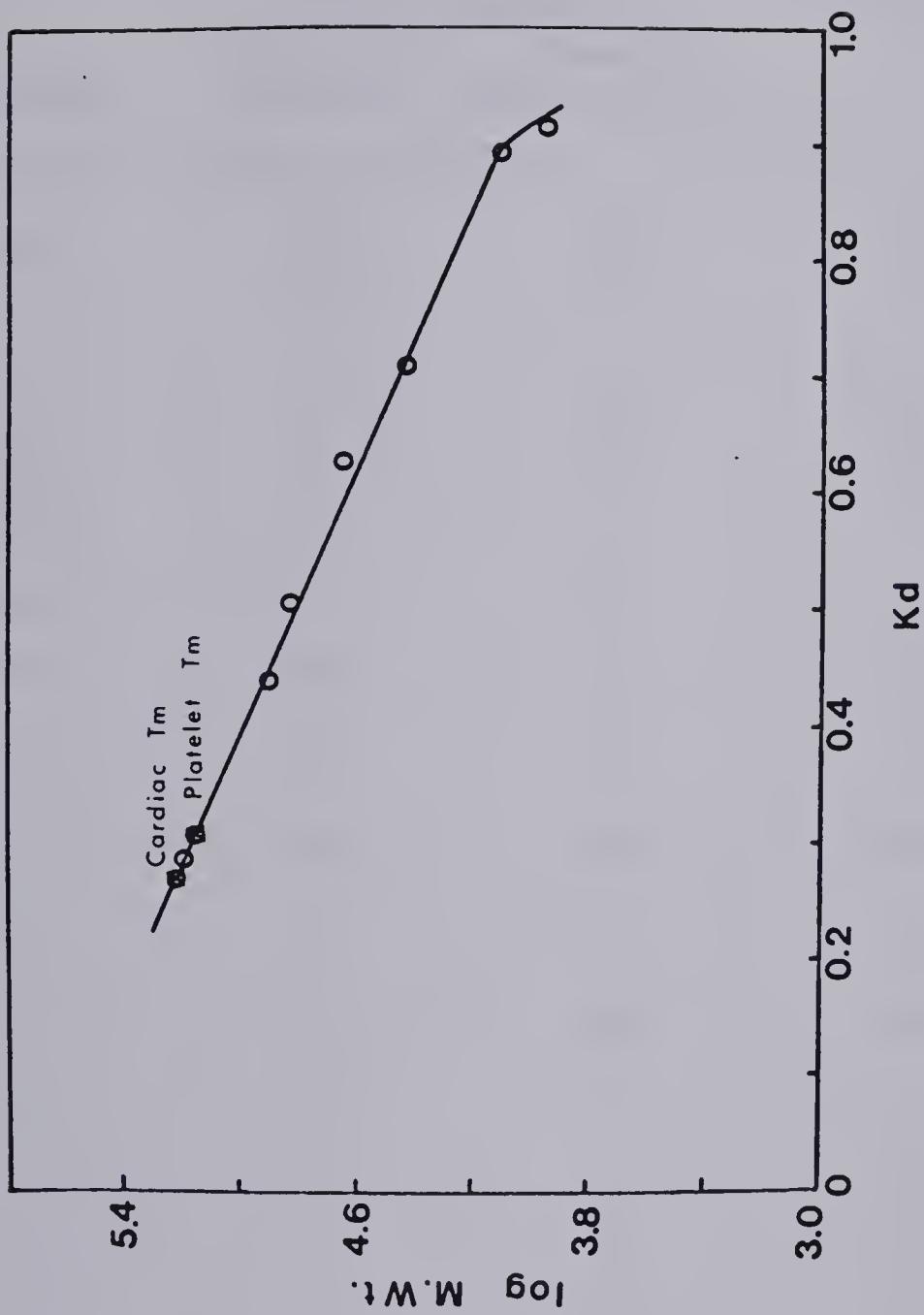


Fig. 24. A plot of the log of the M.Wt. as a function of K_d for proteins run on a Bio-Gel A-1.5m column in 1.0M NaCl, 1 mM DTT, 10 mM Tris, pH 8.0. The standard proteins (o) and their M.Wts. are, from top to bottom, IgG (155,000), creatine phosphokinase (80,000), bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen (25,700), ribonuclease (12,700) and lima bean trypsin inhibitor (9,200). M.Wts. are taken from Fasman (1976).

TABLE VI

Amino Acid Composition of Horse Platelet, Human Platelet and
Rabbit Skeletal Tms (residues/mol of polypeptide chain)

Residue	Amino acid analysis	Horse Platelet Tm	Summation from sequence	Human ^a Platelet Tm	Rabbit ^b Skeletal Tm
Glu	69.7	68 ^c		72	70
Asp	22.4	23 ^d		25	29
Lys	26.8	25		22	39
Arg	19.4	18		17	14
His	2.5	2		1	2
Thr	7.6	7		7	8
Ser	7.2	7		7	8
Gly	7.2	8		9	3
Ala	29	30		29	36
Val	9.9	10		10	9
Met	4.6	4		6	6
Ile	9.4	9		10	12
Leu	28.7	30		36	33
Tyr	3.3	3		2	6
Phe	1.2	1		2	1
Pro	0	0		0	0
Cys	2	2		1.4	1
Total	251	247		257	284

^a Recalculated from Cohen and Cohen (1972) to a M.Wt. of 28,500.

^b Taken from the known sequence of skeletal α Tm (Stone et al., 1974).

^c This figure includes 14 glutamine residues.

^d This figure includes 8 asparagine residues.

half as much tyrosine and serine.

Cysteine, determined as cysteic acid after performic acid oxidation, was present as two residues per platelet Tm subunit, based on a value of 29 alanine residues per chain (Table VII). The results obtained appeared to be slightly high. Control experiments performed on cardiac Tm, which has one cysteine, gave values of around 1.2.

D. ULTRAVIOLET ABSORPTION

The presence of tryptophan in the platelet molecule was ruled out by measuring the absorption spectrum in 0.1M NaOH, and using the values given by Goodwin and Morton (1946). In addition, the platelet Tm's absorption spectrum at neutral pH was virtually identical to that of skeletal Tm, which contains no tryptophan, and to that of the amino acid tyrosine (Bailey et al., 1968). Tyrosine would appear to be the predominate contributor to the ultraviolet absorption spectrum of platelet Tm (Fig. 25).

Both platelet and skeletal Tm exhibit an absorbance minimum in their spectra at 252 nm, which rises to a maximum at 277 nm and then drops off quickly again at higher wavelengths, with an inflection at 282 nm. N-acetyl-tyrosine ethyl ester has an absorbance maximum at 274.6 nm in water, which is red shifted to 278.4 nm in the less polar solvent ethanol (Bailey et al., 1968). The absorbance maximum for the platelet protein therefore indicates that the tyrosines are present in a rather non-polar environment, that is, they are probably involved, at least in part, in forming the hydrophobic core of the molecule.

Nucleic acids, which absorb mainly in the region of 260 nm

TABLE VII

Cysteic Acid Content of Platelet and Cardiac Tm

Protein	Cysteic Acid (nm)	Alanine ^a (nm)	Cysteic Acid (10)Alanine	Cysteic Acid ^b (residues/mol)
Platelet Tm	85.1	101.6	0.837	2.34
	59.0	86.7	0.680	1.97
	125	161	0.775	2.25
Cardiac Tm	49.4	145	0.344	1.24
	27.3	80.9	0.377	1.21

^a

The samples used for the alanine determinations were the same as those used for the cysteic acid determinations, but diluted 10 times.

^b

Calculated from the number of alanine residues per mole of polypeptide chain (29 for platelet and 36 for cardiac Tm).

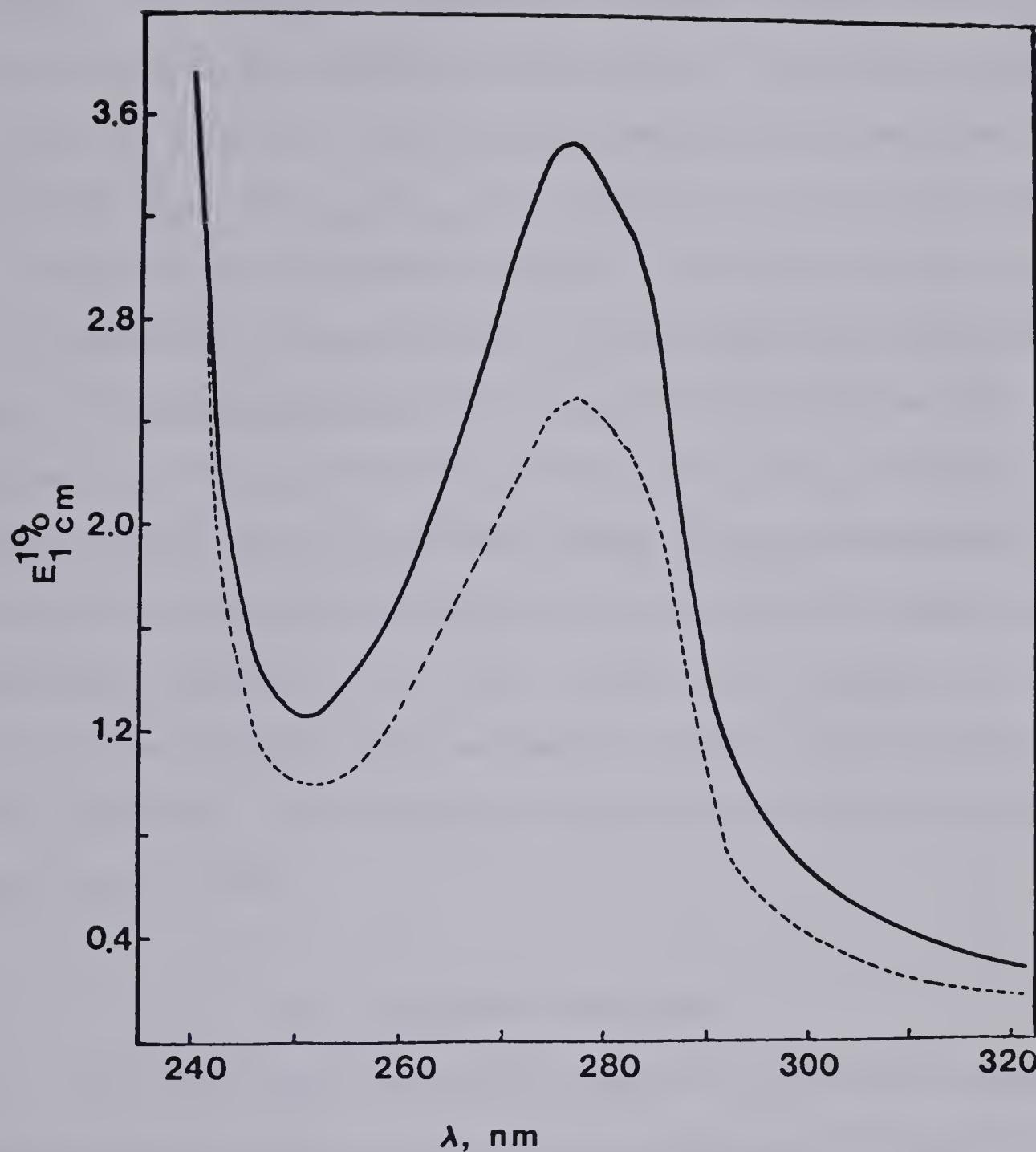


Fig. 25. Ultraviolet absorbance spectra for platelet Tm (prepared by method 2) (---), and skeletal α Tm (—), recorded on a Cary 118c spectrophotometer. Samples were dialyzed overnight against 0.1 M KCl, 1 mM DTT, pH 7.5. The baseline was set using the dialysate. Values are recalculated to $E_{1\text{cm}}^{1\%}$ using $E_{280}^{1\%}$ for platelet Tm as 2.4, and for skeletal α Tm as 3.3.

are often a contaminant of the preparations of Tm made from skeletal muscle. The ratio of the absorbance at 280 nm to that at 260 nm can be used to give an indication of the amount of nucleotide present. If only the amino acids tyrosine and phenylalanine contribute to the UV spectrum the A_{280}/A_{260} ratio should be 2.0 for skeletal Tm (6 tyrosines and 1 phenylalanine) and 1.9 for the platelet protein (3 tyrosines and 1 phenylalanine). These values were calculated using the molar absorbances for tyrosine (1,200 at 280 nm, 582 at 260 nm) and phenylalanine (0 at 280 nm, 147 at 260 nm) in water (Fasman, 1975), and are probably somewhat low as the absorbance intensity of tyrosine at 280 nm can increase when in a non-polar environment (Bailey et al., 1968). However, the A_{280}/A_{260} ratio for the platelet protein, which was found to be 1.8, and for skeletal α Tm, which was 1.9, indicates both preparations were relatively free from nucleic acids.

E. EXTINCTION COEFFICIENT

The large number of assays performed in this study required that an efficient method of accurately determining the concentrations of protein solutions be used. The method chosen was to measure the absorption at 280 nm, and for this reason the $E_{280}^{1\%}$ value for platelet Tm was determined.

This was accomplished by dissolving varying amounts of the protein in 20mM $(NH_4)_2CO_3$, centrifuging to remove aggregates and dust, reading the absorbance of the solution at 280 nm and then taking triplicate samples for concentration determination by amino

acid analysis. As $(\text{NH}_4)_2\text{HCO}_3$ is a volatile buffer the samples could be immediately dried down and hydrolyzed without salt interfering in the subsequent amino acid analyses.

The accuracy of the method was checked by performing control experiments with skeletal α Tm. $E_{280}^{1\%}$ was determined to be 3.3 ($E_{276}^{1\%} = 3.6$) for this protein, slightly higher than a previously published value ($E_{276}^{1\%} = 3.3$, Woods, 1969), but below that of a recent determination based on Kjeldahl analysis ($E_{276}^{1\%} = 3.8$, Wegner, 1979). $E_{280}^{1\%}$ for platelet Tm prepared by method 1 was 2.7 and by method 2 2.4 (Fig. 26). The slight difference in absorbance may perhaps indicate that protein prepared by method 2 contained fewer contaminants.

Even the $E_{280}^{1\%}$ figure of 2.4 for platelet Tm is higher than expected based on the protein's tyrosine content. To facilitate the comparison with skeletal Tm $E_{280}^{1\%}$ values are converted to molar extinction coefficients per tyrosine in Table (VIII). The ϵ_{mTyr} for skeletal Tm is slightly higher than the value for the model compound N-acetyl-tyrosine ethyl ester in a non-polar solvent. This fact may possibly be attributed to light scattering. Tyrosine model compounds show no absorbance above 300 nm (Bailey et al., 1968) yet both platelet and skeletal Tm exhibit absorbance above baseline in this region. Turbidity is not a contributing factor as the protein solutions were always centrifuged prior to absorbance measurements and appeared clear. By measuring the apparent absorbance out of the region of true absorbance and extrapolating this correction into the absorbing region the ϵ_{mTyr} value for skeletal

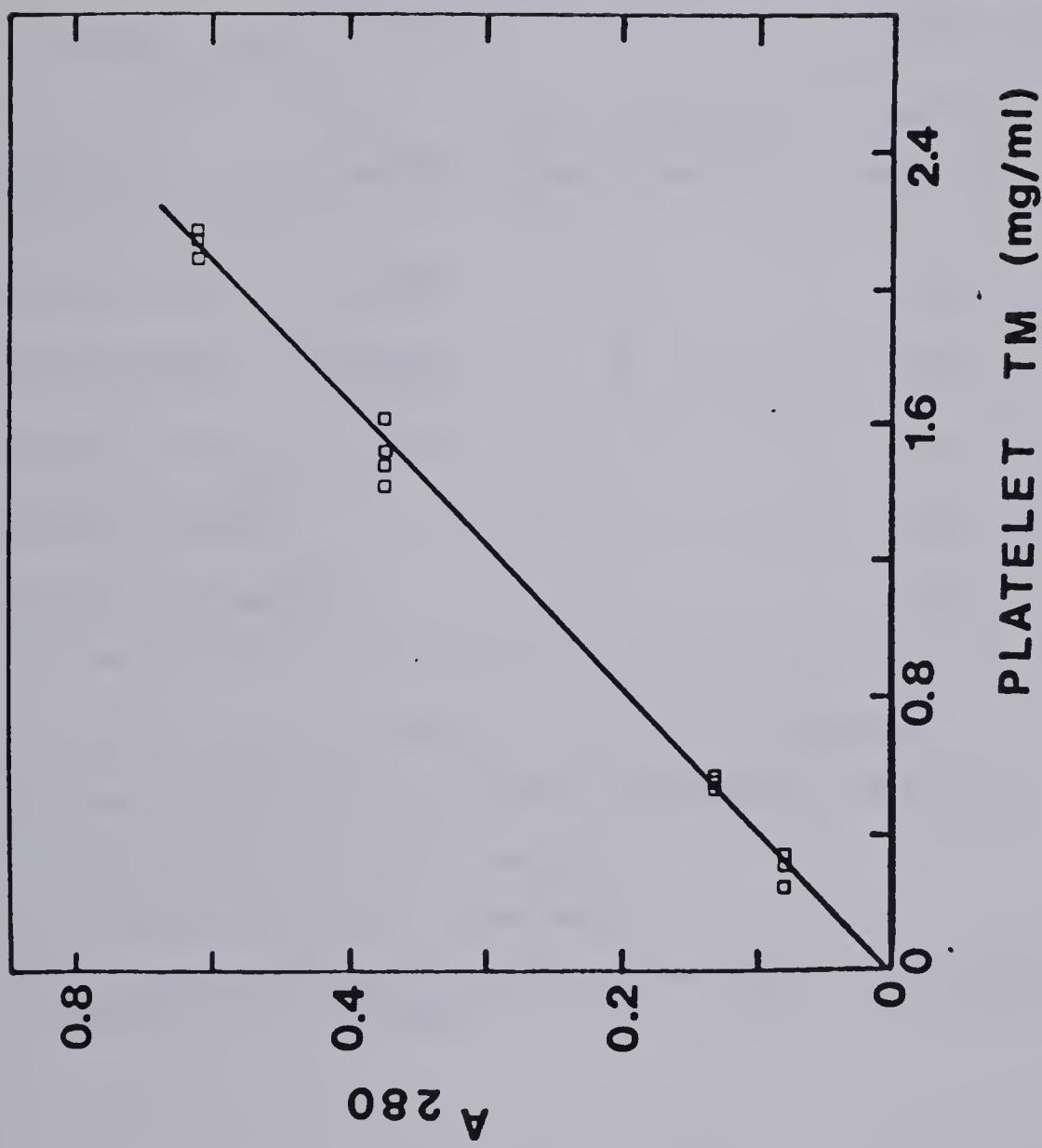


Fig. 26. Determination of $E^{1\%}_{280}$ for platelet Tm prepared by method 2. The figure shows the relationship between the protein concentration (as determined by amino acid analysis) and the absorbance at 280 nm. The slope, calculated by least squares analysis, is 0.237.

TABLE VIII

The Molar Extinction Coefficients per Tyrosine (ϵ_m Tyr)
at 280 nm for Platelet and Skeletal α Tm

Material	ϵ_m^a 280	# of Tyrosines per mole Tm	Calculated ϵ_m Tyr 280	Corrected ^b ϵ_m Tyr 280
Platelet Tm ^c	15,390	6	2,560	2,360
Platelet Tm ^d	13.680	6	2,280	2,090
Skeletal α Tm	21,780	12	1,810	1,660
Tyrosine (H ₂ O) ^e			1,200	
Tyrosine (ethanol) ^e			1,700	

^a The extinction coefficient per mole of protein.

^b Corrected by subtracting the absorbance at 320 nm.

^c Platelet Tm prepared by method 1.

^d Platelet Tm prepared by method 2.

^e These values for N-acetyl-tyrosine ethyl ester are taken from Bailey et al., (1968).

T_m can be made to closely approximate the expected value (Table VIII).

The more important discrepancy, however, that is the higher ϵ_{mTyr} value of platelet T_m as compared to the skeletal protein, cannot be explained in this fashion. Either impurities present in the platelet T_m preparation are absorbing at 280 nm or the tyrosines of this protein have abnormally high absorbances.

Nevertheless, the uncorrected $E_{280}^{1\%}$ values for the skeletal and platelet proteins, which can be accurately converted into concentrations, were used for this purpose in all experiments.

F. CIRCULAR DICHROISM MEASUREMENTS

1. Far Ultraviolet

In general a protein is considered to be composed of three types of secondary structure, α -helix, β -sheet and random coil, each of which has its own characteristic circular dichroism (CD) spectrum. The amount of each form present in a particular protein can be calculated from that protein's CD spectrum (Chen et al., 1974). CD studies on skeletal T_m have indicated that it has an α -helical content (f_H) of between 88 and 97% (Table IX), an amount that few other proteins come close to matching. The far UV CD spectrum of skeletal T_m is thus quite characteristic of the molecule and very similar to that of a pure α -helix. A CD spectrum of the platelet protein is shown in Fig. 27, and corresponds very closely to the spectrum of skeletal muscle T_m .

The magnitudes of the CD minima at 210 and 222 nm were used to calculate the f_H value of the platelet protein as described in methods, producing values of between 80 and 98% (Table IX). The

TABLE IX

Comparison of f_H Values for Skeletal and Platelet Tm

Protein	Refer- ence	Temp. (°C)	Buffer [KCl] M	$[\Theta]_{222}$	f_H^{a} H222
Skeletal Tm	1	27	0.6	-38,940	0.97
	1	27	0.01	-38,940	0.97
	2	25	0.04	-38,800	0.97
	3	10	0.1	-36,400	0.92
	4	-	0.002	-36,000	0.91
	4	-	0.3	-34,600	0.88
Platelet Tm ^b	5	25	0.1	-38,900	0.98
	5	25	0.3	-35,400	0.90
	5	25	0.03	-35,400	0.90
	5	10	1.1	-33,190	0.85
	5	25	1.1	-29,206	0.75

1. Oikawa et al., (1968)

2. Wu and Yang (1976)

3. Pato (1978)

4. Nagy (1977)

5. This study.

^a f_H is calculated from $[\Theta]_{222}$ as described in Chapter II.

^b Concentrations of platelet Tm used to calculate $[\Theta]_{222}$ were determined by amino acid analysis.

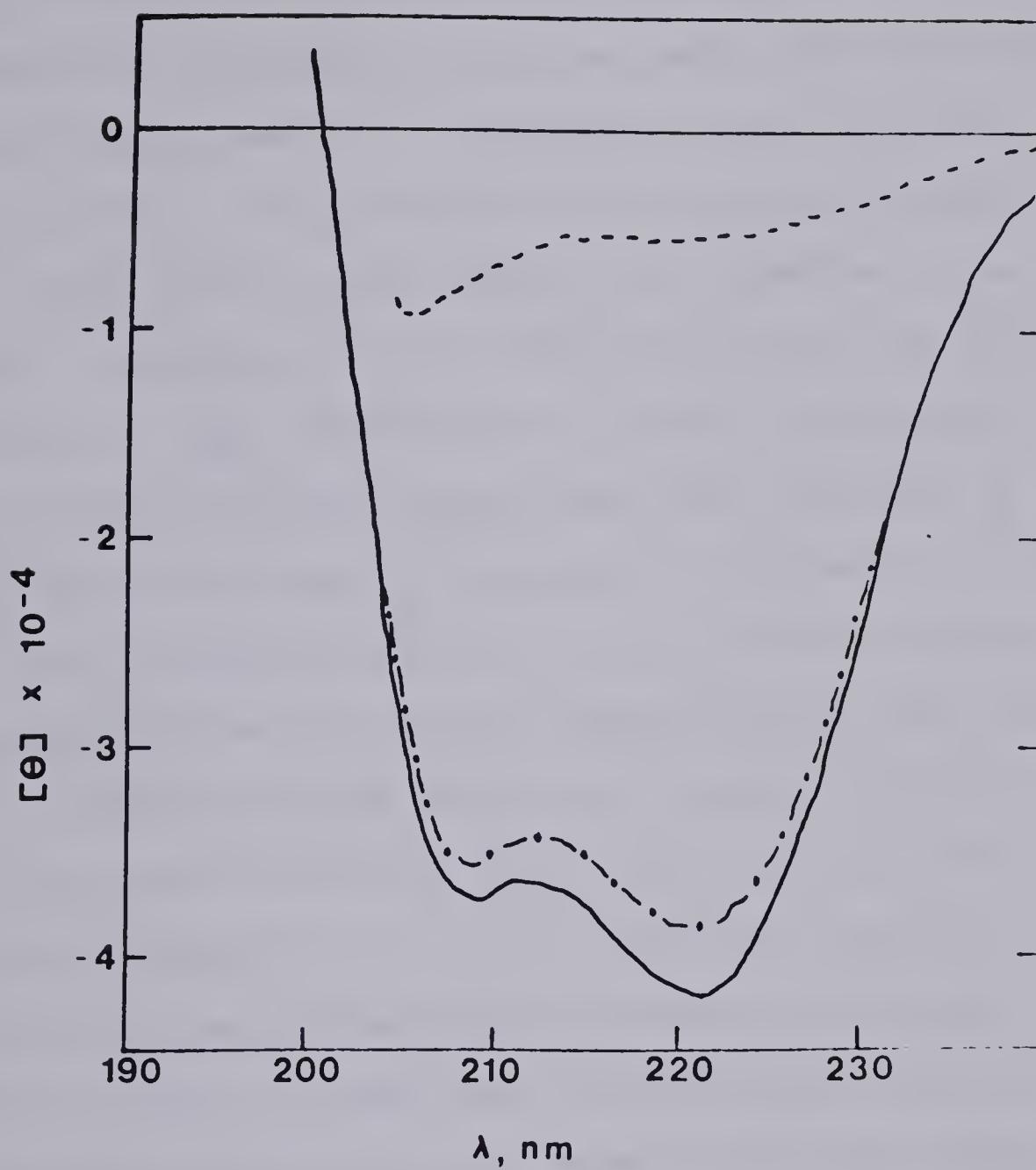


Fig. 27. Far ultraviolet circular dichroism spectra of platelet Tm at different temperatures, in 0.1M NaCl, 20 mM Phosphate, 1 mM DTT, pH 7.0. The temperatures are 8.8°C (—), 25.8°C and recooled to 26°C (↔), and 70.2°C (---).

major source of error in these measurements is in determining the exact concentration of the protein in the solution being examined. Particular difficulty is encountered when the protein is in a solution containing high amounts of salt, as samples cannot be directly taken for amino acid analysis. Concentration errors may be the explanation for the low f_H values found for the platelet protein in 1.1M KCl, since results by both Oikawa et al., (1968) and Wu and Yang (1976) indicate that f_H for skeletal T_m does not vary with ionic strength. Nagy (1977), however, observed a decrease in α -helicity for T_m as the ionic strength was raised (Table IX), so it was possible that the lower f_H values found in high salt for platelet T_m were real. To check this possibility platelet T_m was dialyzed overnight against low ionic strength buffer (30 mM KCl) and 1 ml diluted 1:1 with the dialysis buffer while 1 ml was diluted 1:1 with the same buffer containing 2M KCl. The CD spectra of the two samples were identical in all respects, indicating that ionic strength did not perturb the α -helical content of the protein. The same type of experiment showed that no conformational change took place in platelet T_m when the MgCl₂ concentration of the solution was raised from 3.5 mM to 9.7 mM.

One last experiment, not dependent on concentration, suggests that the platelet protein is probably completely α -helical. A sample of platelet T_m, in 0.1M NaCl buffer, was diluted by a factor of 5 with trifluoroethanol. Such a weakly protic solvent is helix inducing because of its decreased hydrogen bonding capacity as compared to water (Singer, 1962). If any part of the platelet molecule

in a random coil formation could be induced to form an α -helix $[\Theta]_{222}$ would increase. In 80% trifluoroethanol, however, the $[\Theta]_{222}$ value actually decreased slightly, indicating that all regions of the protein capable of forming an α -helix were already in a helical conformation.

An indication of the stability of the coiled coil formed by T_m can be obtained by measuring the loss in α -helical content, reflected in the $[\Theta]_{222}$ value, as the temperature is raised. The platelet protein was 50% denatured at a temperature of 47°C in a buffer containing 0.1M KCl and at 51°C in 1.0M KCl (Fig. 28). Experiments, performed in our lab, demonstrated that the midpoint of the melting temperature curve for skeletal α - T_m occurred at 44°C in 0.1M KCl and 51°C in 1.0M KCl (Pato, 1978). In both cases the denaturation of the platelet protein, which was reversible, took place in one step, indicating a uniform structural stability throughout the molecule.

From these CD results the platelet protein appears to be very similar in structure and stability to skeletal α T_m . In fact it is more stable than a number of muscle T_m s, for example scallop T_m which is one-half denatured at 30.8°C in 0.2M KCl (Woods, 1976).

The CD results are strong evidence for the coiled-coil T_m -like nature of the platelet protein.

2. Near Ultraviolet

The CD spectrum of a protein can also be measured in the near UV. The CD spectrum in this region, due to aromatic residues and disulfide bonds, has an extremely small intensity as compared to those of peptide chromophores in the far UV. For skeletal and

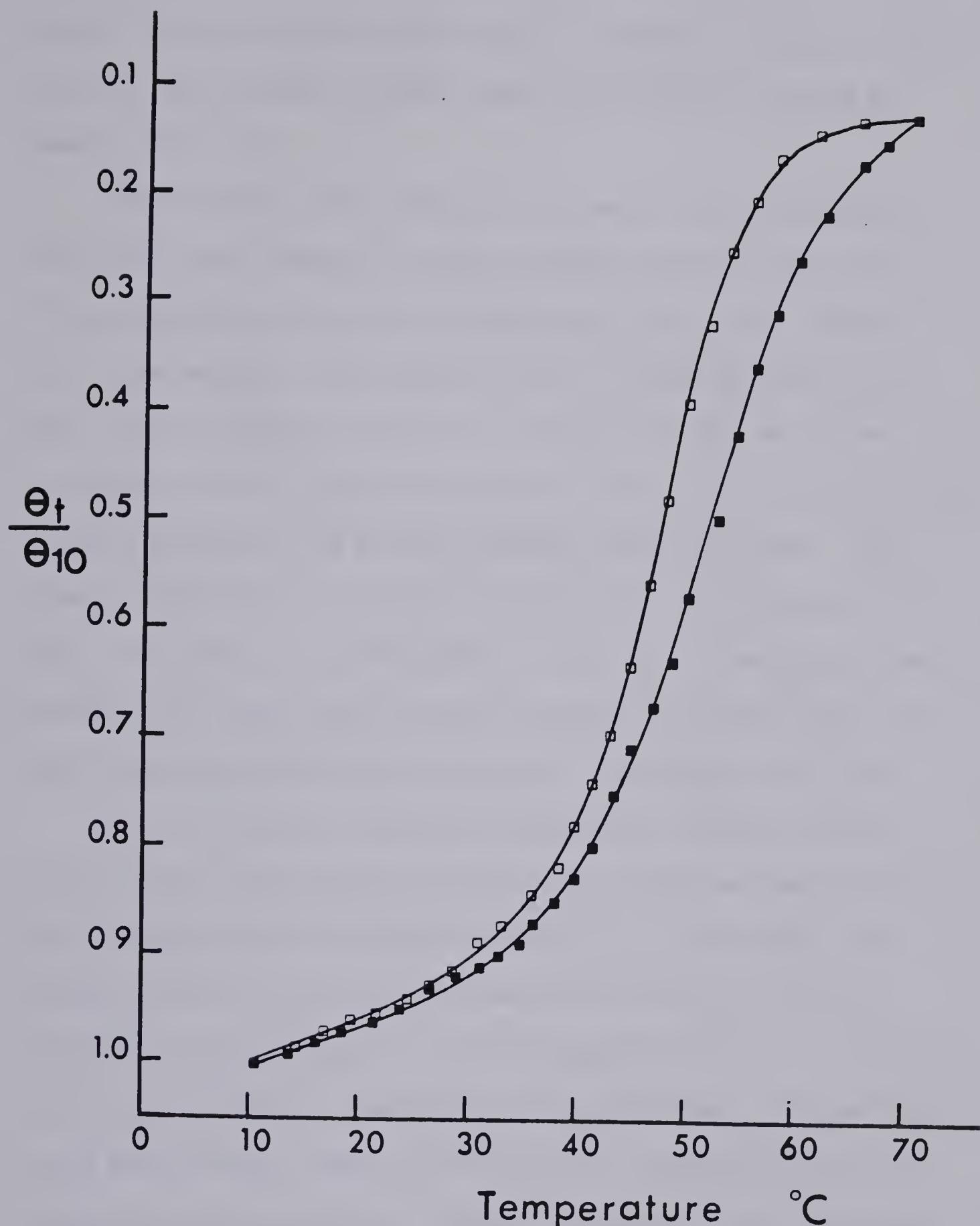


Fig. 28. CD thermal denaturation curves of platelet Tm in 1.0M NaCl (■), and 0.1M NaCl (□), 50 mM Phosphate, 1 mM DTT, pH 7.0. The ratio $\frac{\Theta_t}{\Theta_{10}}$ is the fractional ellipticity observed at the indicated temperature, with respect to the ellipticity at 10°C, at 221 nm.

platelet T_m the near UV spectra can be considered as arising almost entirely from tyrosine residues, once the disulfide bridges are reduced (Fig. 29).

The intensity of the skeletal T_m near UV CD per tyrosine is relatively large compared to that of other proteins and is most likely caused by the specific interaction of tyrosines that are very close together (rings separated by <0.8 nm) (Bullard et al., 1976). This situation can occur in T_m only if the two chains are in register, so that the five tyrosines on each chain which contribute to the hydrophobic core are able to pair with each other. $\Delta\epsilon_{mTyr}$ values, taken from the literature for skeletal T_m, are given in Table (X), as are values determined in this study for platelet and skeletal T_m. These are difficult to measure accurately due to the small ellipticity values and consequent interference from noise.

If it is assumed, as would be expected, that only the ten core tyrosines contribute to the skeletal T_m CD spectrum while all six tyrosines contribute to the platelet CD (as described later sequence studies place all tyrosines in platelet T_m into core positions), then the rotary strengths observed for the two proteins are in good agreement, considering the large errors which may occur during measurement. These results are most interesting for they suggest that the two subunit chains of platelet T_m may be aligned in register with each other.

Some fine structure in the platelet T_m spectrum, observable at around 260 nm, can be attributed to phenylalanine (Nagy, 1977).

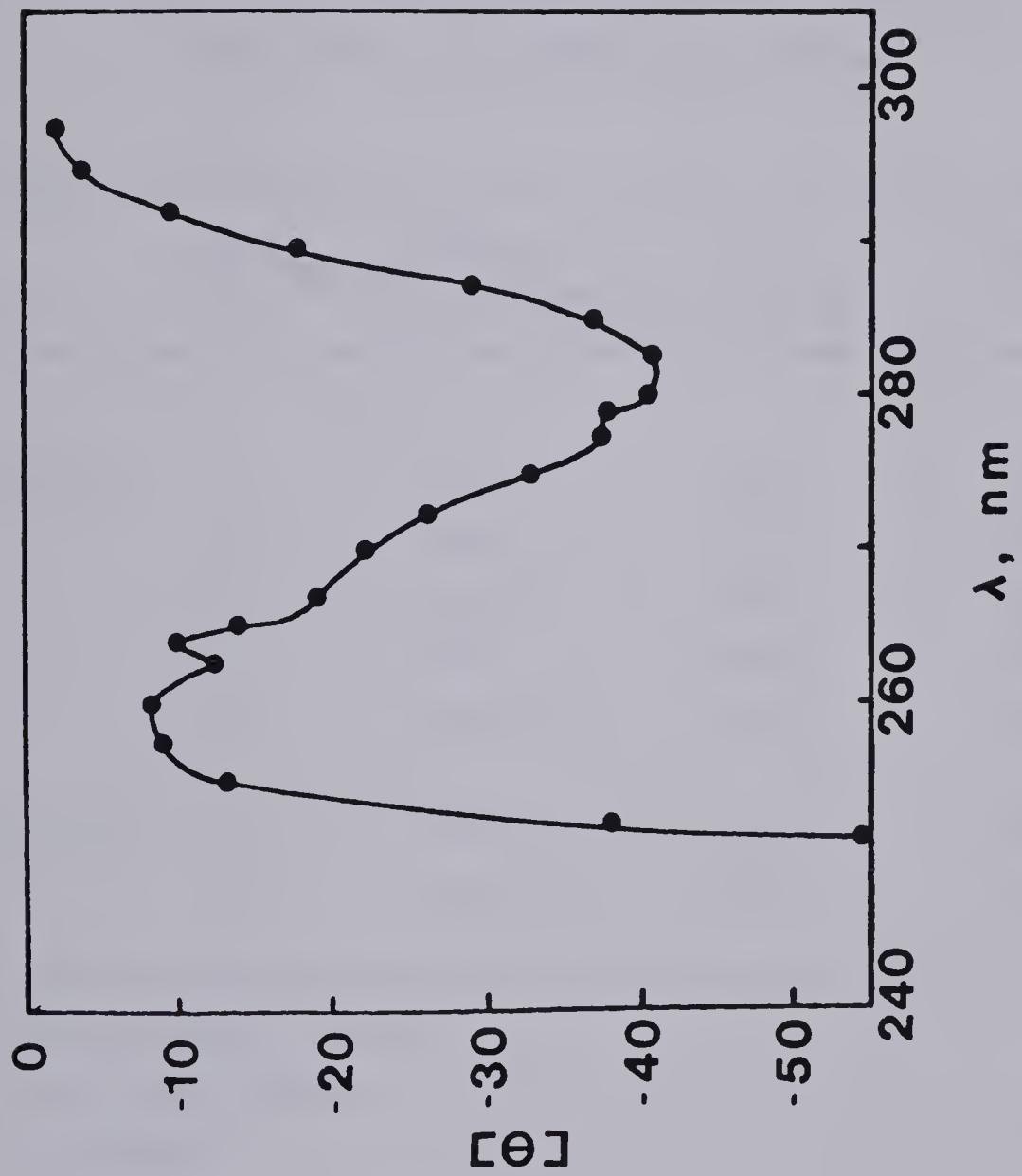


Fig. 29. The near ultraviolet circular dichroism spectrum of platelet Tm, measured in 30 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 10 mM Tris, pH 7.5.

TABLE X

Intensity of the Circular Dichroism Spectra of
 Platelet and Skeletal Tm at 282 nm

Protein	Refer- ence	$[\Theta]_{282}$ (deg. \cdot cm 2 /dmole)	$\Delta\epsilon_m$ (1/M \cdot cm)	$\Delta\epsilon_m$ Tyr ^a (1/M \cdot cm)
Skeletal Tm	1	-44.9	-7.8	-0.78
	2	-38	-6.6	-0.66
	3	-27.6	-4.8	-0.48
	4	-46	-8.0	-0.80
	5	-39 ± 6	-6.8	-0.68
Platelet Tm	6	-32 ± 6	-4.9	-0.82
	7	-40 ± 6	-5.9	-0.98

1. Bullard et al., (1976)
2. Wu and Yang (1976)
3. Nagy (1977)
4. Chao and Holtzer (1975)
5. This study.
6. This study. Platelet Tm prepared by method 2.
7. This study. Platelet Tm prepared by method 1.

^a The difference in molar extinction coefficient per tyrosine was calculated assuming 10 tyrosines in skeletal Tm and 6 in platelet Tm contribute to the CD spectrum.

G. PARACRYSTALS

Skeletal Tm, when dialyzed against divalent cations at pH 7 to 9, precipitates to form ordered aggregates termed paracrystals or tactoids (Cohen & Longley, 1966). The coiled-coils lie parallel to the long axis of the paracrystal and are bound side by side to each other through bridges formed by aspartyl and glutamyl carboxyl interacting with the divalent cation. When stained with uranyl acetate and examined in the electron microscope the skeletal Tm paracrystals display a regular banding pattern in the longitudinal direction with a periodic repeat of 39.5 ± 0.5 nm (Caspar et al., 1969; Hurwitz & Walton, 1977). This is close to the molecular length of skeletal Tm estimated from the unit cell diagonal in crystals, reported to be 41.0 ± 0.4 nm (Cohen et al., 1973). It can be calculated though that for a perfect coiled-coil consisting of 284 residues per chain, the head to tail distance should be 42.3 nm (Johnson & Smillie, 1975). The discrepancy between this value and the one determined from the crystals can be accounted for by assuming an 8 to 9 residue overlap between the amino and carboxyl terminal ends of Tm when arranged in the filaments of the crystal. The lower value of 39.5 nm in the paracrystals may be due to the plaiting of the antiparallel array of Tm filaments in these structures.

Platelet Tm did not form paracrystals as readily as readily as muscle Tm. Even at a platelet Tm concentration of 5 mg/ml the yield of tactoids was low, and those that were produced were either quite short (Fig. 30a) or longer but very narrow in diameter (Fig. 30b). At this protein concentration tactoids produced by skeletal Tm are so large that they are difficult to view in the electron microscope.

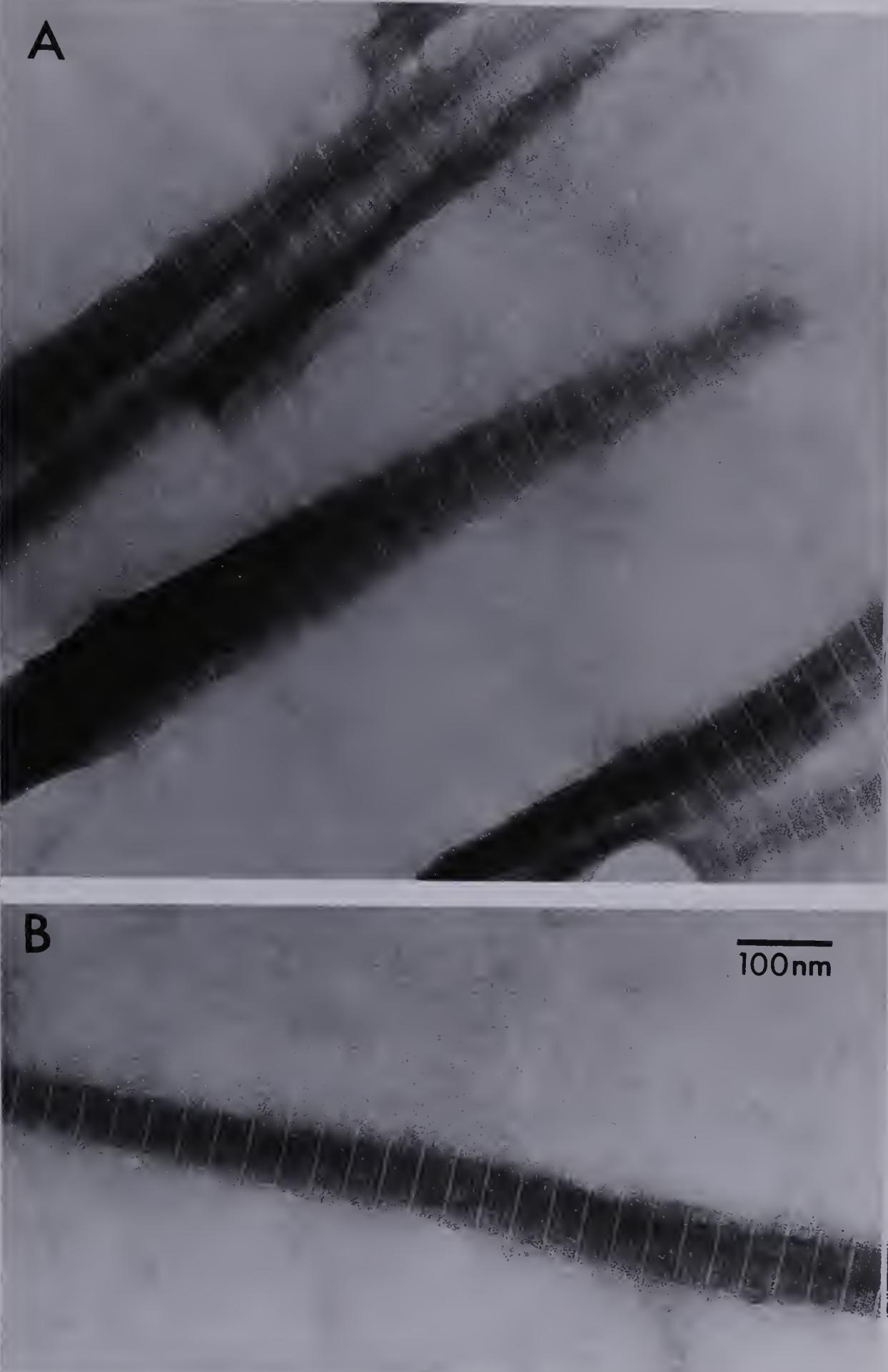


Fig. 30a,b. Paracrystals of platelet Tm as viewed in the electron microscope. Paracrystals were grown by dialysis against 50 mM $MgCl_2$, 50 mM Tris pH 8.0, stained with 1% uranyl acetate, and viewed at an enlargement of 48,000 x.

Periodicities of 4.55 nm for platelet T_m and 5.40 nm for skeletal T_m were measured directly from micrographs (photographed using identical magnifications) of micrographs. Taking a muscle T_m repeat of 39.5 nm results in a period of 33.3 ± 0.8 nm for the platelet molecule. The ratio of these distances (1.19) corresponds very well with the ratio of the M.Wts. (1.16) of the two proteins. This means that the length to mass ratios of the two proteins in the paracrystal are virtually identical, giving further evidence for the structural similarity between them, and for the completely coiled-coil nature of the platelet molecule.

A coiled-coil of 57,000 daltons would be expected to have a length of 36.4 nm, so we can assume that in the paracrystal there is an overlap between the amino and carboxyl termini of the platelet protein. The smaller size of the platelet T_m paracrystals and the greater difficulty in preparing them may be a consequence of a weaker interaction between the two ends of the molecule. (For further discussion see Chapter V, Viscosity experiments.) Skeletal T_m which no longer polymerizes, as a result of treatment with carboxypeptidase, is still able to form paracrystals (Ueno et al., 1976).

The banding pattern of the platelet T_m paracrystals is quite distinct from that of muscle T_m, indicating differences in the distribution of negative charges in the two proteins. The platelet paracrystals exhibit a symmetrical pattern consisting of a dark middle band surrounded on either side by a thinner, lighter band and then by a very narrow white band. A similar pattern was observed

with paracrystals formed from human platelet Tm, which displayed a repeat distance of 34.3 ± 0.5 nm (Cohen & Cohen, 1972).

Further analysis will be needed in order to determine how the platelet molecules are packed and orientated within the paracrystal and how the banding pattern correlates with the amino acid sequence of the protein.

H. SEQUENCE ANALYSIS

After a purification procedure for platelet Tm had been developed, the sequence analysis of the protein was undertaken in our laboratory by Dr. W.G. Lewis. The studies were performed on a mixture of α and β platelet Tm with a ratio of α to β of about 1 to 2.

Dr. Lewis citraconylated the lysine residues of the platelet protein and then used trypsin to cleave at the arginine residues. This procedure yielded one large fragment (TA), whose sequence I determined.

1. Preparation of Fragment TA

Platelet Tm (120 mg) was carboxymethylated as described by Crestfield et al., (1963), dissolved in 15 ml 50 mM PO_4 , pH 8.0, and citraconylated with a 40 fold molar excess of citraconic anhydride over lysines. After desalting on a Sephadex G-25 column the freeze dried product was dissolved in 0.2M $(\text{NH}_4)\text{HCO}_3$, pH 8.0 and digested with 1.2 mg TPCK treated trypsin at 37°C for two hours. The reaction was terminated with 1 mM diisopropylfluorophosphate and the product lyophilized. Following decitraconylation in 5% formic acid for two hours at 45°C the sample was applied to a Sephadex G-75 (200 x 2.5 cm) column equilibrated with 5% formic acid. The first fraction to

elute (fig. 31) was termed TA and was homogeneous as determined by its amino terminal sequence and its migration as a single band on high voltage paper electrophoresis at pH 6.5.

2. Sequence of TA

Intact TA (200 nmol) was subjected to automatic sequence analysis on a Beckman Model 890 B sequencer. This resulted in the sequence of the first ten residues of the fragment (Table XI) and indicated that homology existed between TA and a region of skeletal α Tm beginning at Ala - 183. Inspection of this part of the skeletal α Tm sequence indicated that digestion of the decitraconylated TA with trypsin, to cleave at the lysine residues, would produce a number of peptides that could easily be separated by high voltage paper electrophoresis on the basis of charge and size.

TA (750 nmol) was thus dissolved in 4 ml of 0.2M $(\text{NH}_4)_2\text{HCO}_3$, pH 7.8 and digested with 75 nmol of TPCK treated trypsin (Worthington) for 4 hours at 37°C. The digestion was terminated by lyophilization and the resulting peptides were separated by preparative paper electrophoresis at pH 6.5. The peptides, designated T1 to T11, were eluted from the paper and submitted to amino acid analysis. T5, the neutral band, was not eluted until after paper electrophoresis at pH 3.5, which resolved two peptides, T5a and T5b. The amino acid analyses, yields and mobilities of the tryptic peptides are summarized in Table (XII).

For most peptides the manual sequence analysis, carried out as described in the methods section, was straightforward and is summarized in Table (XII). Complications that arose with certain peptides are described below.

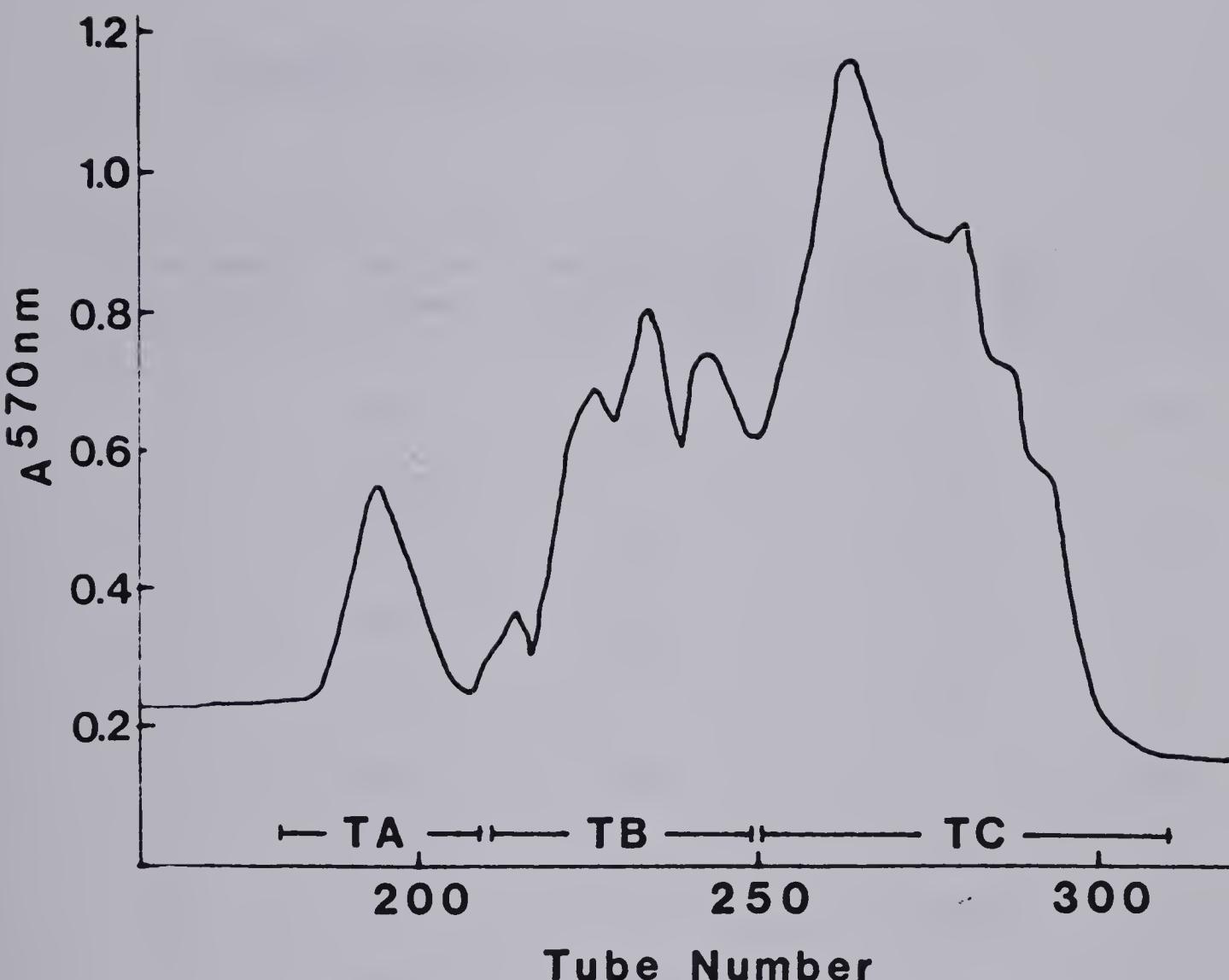


Fig. 31. Fractionation of a tryptic digest of citraconylated platelet Tm (120 mg) on a Sephadex G-75 column (2.5 x 200 cm) run in 5% formic acid. Peak TA was pooled as indicated. Column effluent was monitored by ninhydrin analysis after alkaline hydrolysis.

TABLE XI

Automated Sequence Analysis of Fragment TA

Step of Edman ^a Degradation	Residue ^b Number	Residue Ident- ^c ified by GLC	Residue Ident- ^d ified by TLC	Yield (nmole)
1	183	Ala	Ala	78
2	184		Glu	
3	185	Val	Val	92
4	186	Ser	Ser	
5	187		Glu	
6	188	Leu		69
7	189		Lys	
8	190		CM-Cys	
9	191	Gly	Gly	11
10	192		Asp	

^a The automated Edman degradation of 200 nmole TA was carried out in a Beckman 890B Sequencer using Beckman program # 50972 (Edman & Begg, 1967).

^b The residue numbering is that of skeletal α Tm (Stone & Smillie, 1978).

^c The phenylthiohydantoin derivatives were identified by gas-liquid chromatography (Pisano & Bronzert, 1969).

^d The phenylthiohydantoin derivatives were identified by both one dimensional (Edman, 1970) and two dimensional (Summers et al., 1973) thin layer chromatography.

TABLE XII

A Summary of the Properties and Sequence Data of
Peptides Derived from Tryptic Digestion of TA

Peptide	Yield % ^a	Mobility ^b	Net Charge ^c	Purification ^d	Amino acid Composition and Sequence Data ^f
T1	50	+0.59	+1	6.5	Leu-Lys 1.0 1.0
T2	7	+0.4		6.5	Leu-(Asx,Thr,Ser,Glx,Gly,Val,Lys) 1.0 0.4 0.7 0.7 1.1 0.6 1.0 2.2
T3	25	+0.32	+1	6.5,1.8	Leu-(Asx,Ser,Glx,Gly,Leu,Lys) 0.8 1.1 1.4 0.6 0.8 1.6 2.0
T4	45	+0.26	+1	6.5	Asn-Val-Thr-Asn-Asn-Leu-Lys 1.0 1.0 1.1 1.0 1.0 1.0 1.0
T5a	45	0	0	6.5,3.5	Leu-Leu-Ser-Asp-Lys 0.8 0.8 1.2 1.0 1.0
T5b	12	0	0	6.5,3.5, 1.8	Tyr-Ser-Glu-Lys 0.9 1.1 1.2 1.0
T6	12	-0.06		6.5	Asx-Val-(Asx,Thr,Leu,Lys) 0.8 1.0 1.6 0.9 1.0 1.1
T7a+b	70	-0.31	-1	6.5	Ala-Glu-Val-Ser-Glu-Leu-Lys 0.9 1.0 1.0 0.9 1.0 1.0 1.0 Ser-Leu-Glu-Ala-Ala-Ser-Glu-Lys 0.9 1.0 1.0 0.9 0.9 0.9 1.0 1.0
T8	51	-0.38	-1	6.5	Glu-Ala-Glu-Thr-Arg-(Asp,Ser,Gly) 1.0 1.1 1.0 1.0 1.0 0.2 0.2 0.2
T9	49	-0.46	-3	6.5	Tyr-Ser-Glu-Lys-Glu-(Asp,Lys,Tyr, 0.9 1.1 1.2 1.0 1.2 1.1 2.0 0.9 Glu,Ile) 3.6 0.9
T10	21	-0.62	-3	6.5,1.8	Glu-Asp-Lys-Tyr-Glu-(Glu,Ile,Lys) 0.9 0.9 1.0 1.2 0.9 1.8 0.8 1.0
T11	67	-0.86	-4	6.5	CmCys-Gly-Asp-Leu-Glu-Glu- 0.4 1.0 1.1 1.0 1.0 1.0 1.0 1.0 Leu-Lys 1.0 1.0
T7-2i	25 ^g	0	0	6.5	Val-(Ser,Glu,Gly,Ala,Leu,Lys) 1.0 1.1 1.2 0.2 0.2 1.0 1.0

TABLE XII (Continued)

Peptide	Yield % ^a	Mobility ^b	Net Charge ^c	Purification ^d	Amino acid Composition and Sequence Data ^f
T7-2ii	14 ^g	-0.37	-1	6.5	Pooled with T7-2iii
T7-2iii	17 ^g	0.61	-2	6.5	Glu-Ala-Ala-Ser-Glu-Lys 1.0 0.9 0.9 1.2 1.0 1.0 → → → → → →
T3-AM1	20 ^h	+0.8	+2	6.5	Lys-(Ser,Gln,Leu,Lys) 1.0 1.2 0.8 1.1 1.0 → → → → →
T3-AM2	22 ^h	-0.44	-1	6.5	Leu-Leu-(Ser,Asp) 0.8 0.8 1.2 1.0 → → → →

^a Yields of peptides are based on moles of TA taken for digestion.

^b Electrophoretic mobilities are relative to Asp at pH 6.5.

^c Net charges were calculated from the M.Wt. and mobility of the peptide by the method of Offer (1966). Acids and amides could be assigned directly from this information.

^d Purification was by high voltage electrophoresis at the pH indicated.

^e Amino acid compositions are in molar ratios. The notation (→) is for sequence determination by the dansyl-Edman method.

^g Based on the moles T7 taken for the dansyl-Edman steps.

^h Based on the moles T3 taken for A. mellea digestion.

(a) T7

This fraction appeared to be composed of two peptides from the amino terminal analysis, which revealed two spots corresponding to Ala and Ser. The two peptides, T7a and T7b, could not be separated by high voltage paper electrophoresis at either pH 3.5 or 1.8. Fortunately one of the peptides was the amino terminal peptide of TA whose sequence had previously been determined. It was therefore possible to sequence the mixture of T7a and T7b, obtain two dansyl spots at each step, and determine which one belonged to each peptide.

To confirm these results an attempt was made to separate the two peptides by high voltage electrophoresis at pH 6.5 after two Edman steps had been performed, when the amino terminal peptide would have lost a negative charge. Three peptides resulted from this procedure, T7-2i, T7-2ii, and T7-2iii (Table XIII). T7-2ii and T7-2iii were identical except for mobility; presumably T7-2iii differed by having the ϵ -amino group of lysine blocked by PITC. T7-2i corresponded to the amino terminal peptide of Ta. Amino acid analysis and Edman degradation confirmed the sequence of the second peptide.

(b) T3

This peptide, isolated in low yield, gave an amino terminal Leu, and from its mobility and amino acid composition was thought to be the result of an incomplete cleavage between T5a and T1. Attempts to sequence past the amino terminal Leu, were however, unsuccessful.

To confirm the origin of this peptide it was digested overnight at room temperature in 10 mM NaHCO₃, pH 9.0, with Armillaria

mellea protease. This enzyme cleaves on the amino terminal side of lysine (Lewis et al., 1978). Two peptides of the expected mobility were isolated, one of which, T3-AM2, gave a good amino acid analysis. The other, however, although containing the expected ratio of lysine to leucine contained serine and glutamine as well.

These results indicate that the sequence of this peptide must be Leu-Leu-(Ser, Asp)-Lys ---, in agreement with it having been derived from incomplete digestion of the lysine between T5a and T1. However, if the serine and glutamine found in T3-AM1 are not contaminants the sequence past this point may differ from that of T1, indicating heterogeneity in the platelet Tm possibly due to the presence of both α and β components.

(c) T9

T9 is a large peptide stretching from Tyr-214 to Lys-226 and including Lys-217, partial cleavage at which produces T5b and T10, and Lys-220 at which no cleavage occurs. These lysine bonds are probably not susceptible to trypsin as a result of the high concentration of negative charges around them.

T9 could only be sequenced to Glu-118 and T10 only as far as Glu-222. To confirm the carboxyl terminal sequence of this peptide, T9 was subjected to a Staphylococcus aureus protease digest (Houmar and Drapeau, 1972) at pH 7.8 in 0.1M $(\text{NH}_4)\text{HCO}_3$ buffer. Under these conditions the enzyme is specific for cleavage on the carboxyl terminal side of glutamic acid residues. High voltage paper electrophoresis at pH 6.5 revealed that three major and one minor peptide had been produced (Table XIII). The isolation of these

TABLE XIII

Amino Acid Compositions and Yields of Peptides Derived from
an *S. aureus* Protease Digest of T9

Amino acid ^a	T9-S1	T9-S2	T9-S3 ^b	T9-S4	Sum T9-S1,2,4	T9
Asp			1.3	1.0	1.0	1.1
Ser		1.0	1.1		1.0	1.1
Glu		1.9	4.9	2.9	4.8	4.7
Gly			0.4			
Ala			0.2			
Ile	1.0				1.0	0.9
Lys	1.0	1.0	2.0	1.0	3.0	3.0
Tyr		1.0	1.6	1.0	2.0	1.7
Total	2	4.9	11.5	5.9	12.8	12.5
Yield (%) ^c	50	42	6	38		
NH ₂						
terminus	Ile	Tyr	-	Asp		
Mobility ^d	+0.64	-0.34	-0.67	-0.79		
Net charged	+1	-1	-3	-3		

^a Values are given in residues per mole peptide.

^b T9-S3 is a partial digestion product consisting of T9-S2 and T9-S4, and therefore has not been included in the summation of the peptides derived from T9.

^c Based on the moles T9 taken for digestion.

^d Determined as described in the footnotes to Table XII.

peptides, particularly Ile-Lys, confirmed the sequence of T9.

T9-S3 is a partial digestion product of T-9S2 and T9-S4.

(d) T2 and T6

T2, isolated in very low yields, could not be assigned in the sequence.

T6 is almost certainly T4 with an aspartic acid residue in place of one of the three asparagines. Whether this is an indication of true heterogeneity or whether deamidation occurred during isolation of the peptide is not known. Having been isolated in much higher yields T4, rather than T6, was assigned to the sequence.

3. Assignment of Amides

All acids or amides could be unambiguously identified by the mobility of the peptide during high voltage electrophoresis at pH 6.5. This is a fortunate consequence of the fact that no peptide contains both an amide and an acid.

4. Ordering of the Peptide

Overlaps between the tryptic peptides were not determined. Instead, the peptides were ordered based on the known sequence of skeletal muscle α Tm (Fig. 32). The homology between the sequences of the skeletal and platelet protein was so great that such assignments could be made with confidence. Where the position of the peptide was known (ex. T7a, T11, T8) the assignments based on homology were shown to be correct.

The amount of each amino acid present, as calculated from the sequence, is in excellent agreement with the amino acid analysis of TA, indicating that all peptides have been accounted for (Table XIV).

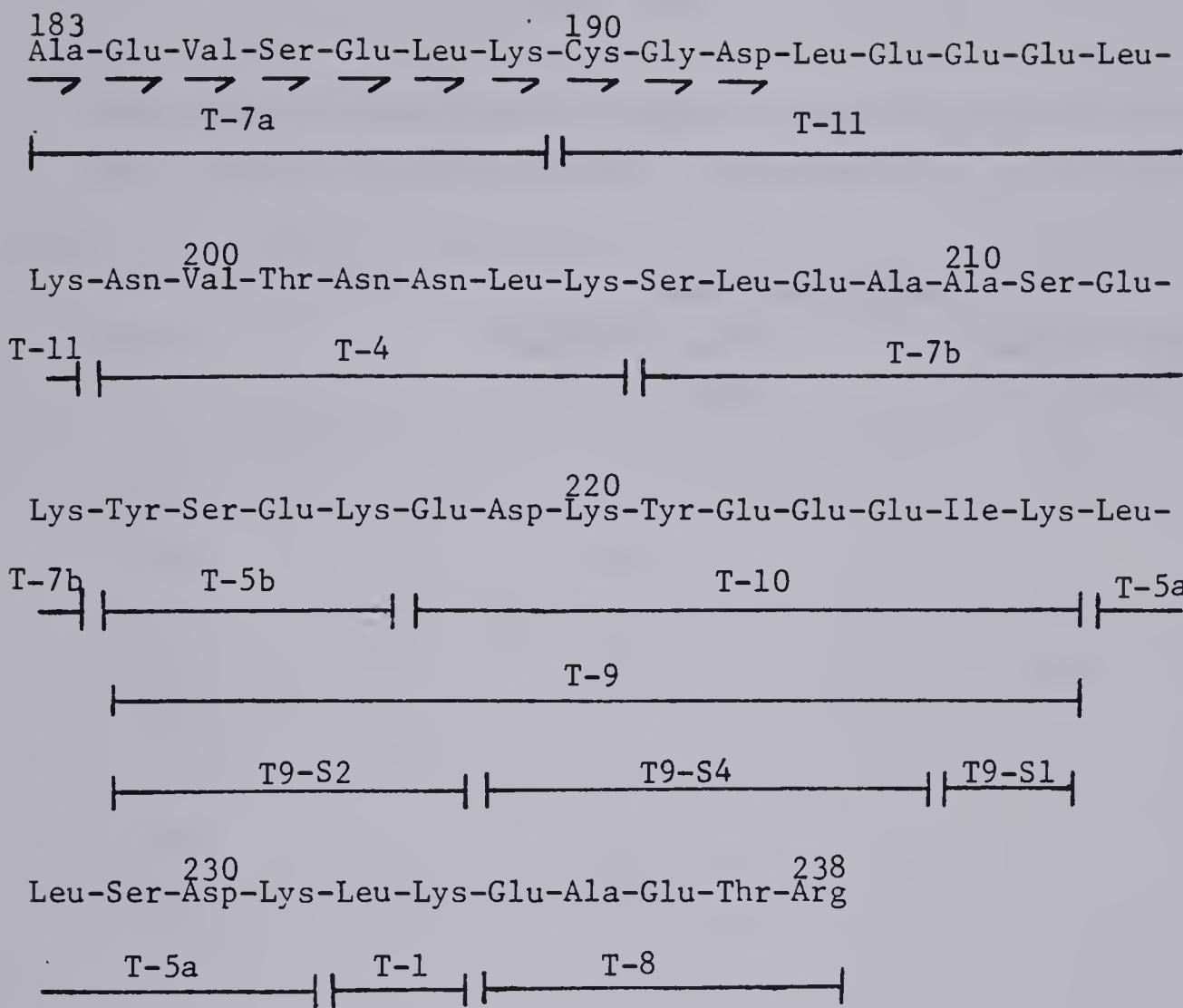


Fig. 32. Summary of evidence for the sequence of fragment TA from platelet Tm. Residues 183 to 192 were established by automatic sequencer (→). Evidence for peptides derived from tryptic or S. aureus protease digestion are given in Tables XII and XIII. The numbering of the residues is based on the sequence of skeletal α Tm (Stone & Smillie, 1978).

TABLE XIV

Comparison of the Amino Acid Composition of TA as Determined from Amino Acid Analysis and by Summation from the Sequence

Residue	Amino acid composition ^b	
	Based on amino acid analysis	Summation from the sequence
Asp	6.3	6
Thr	1.9	2
Ser	4.2	5
Glu	14.2	14
Gly	1.3	1
Ala	4.1	4
Val	1.6	2
Ile	1.0	1
Leu	8.0	8
Tyr	1.9	2
Phe	0	0
His	0	0
Lys	9.1	9
Arg	1.0	1
CmCys ^a	-	1
Total	54.6	56

^a This residue was poorly detected by amino acid analysis.

^b Values given are per mole of TA.

I. DISCUSSION

The entire sequence of platelet Tm, 247 amino acid residues, has recently been completed in our lab by Dr. W.G. Lewis and is shown in Fig. (33). Information deduced from the sequence amply confirms, and extends, the results of the experiments just described. The sequence of platelet Tm will be discussed in this chapter only insofar as it relates to the structure of the protein. Aspects concerned with the ability of the platelet molecule to function in a biological system will be covered in the next chapter.

Much of the sequence of platelet Tm shows striking homology to that of skeletal Tm; only the amino and carboxyl terminal ends of the two proteins differ significantly (Fig. 34). The subunit M.Wt. of platelet Tm, calculated from the sequence, is 28,387, in excellent agreement with the M.Wt. as determined by SDS gel electrophoresis. The platelet molecule is 37 residues (4371 daltons) smaller than the subunit of skeletal Tm. This difference is completely accounted for by one large deletion at the amino terminus.

It has been previously postulated (McLachlan et al., 1975) that skeletal Tm has evolved by the process of repeated gene duplication from a single ancestral 42-residue segment. The nature of the platelet protein deletion is consistent with this concept, which may mean that the platelet molecule is a more primitive form of Tm, in the sense that it has not undergone the last gene duplication event.

Because the sequences of the skeletal and platelet protein are so similar all the features of the former which contribute to the

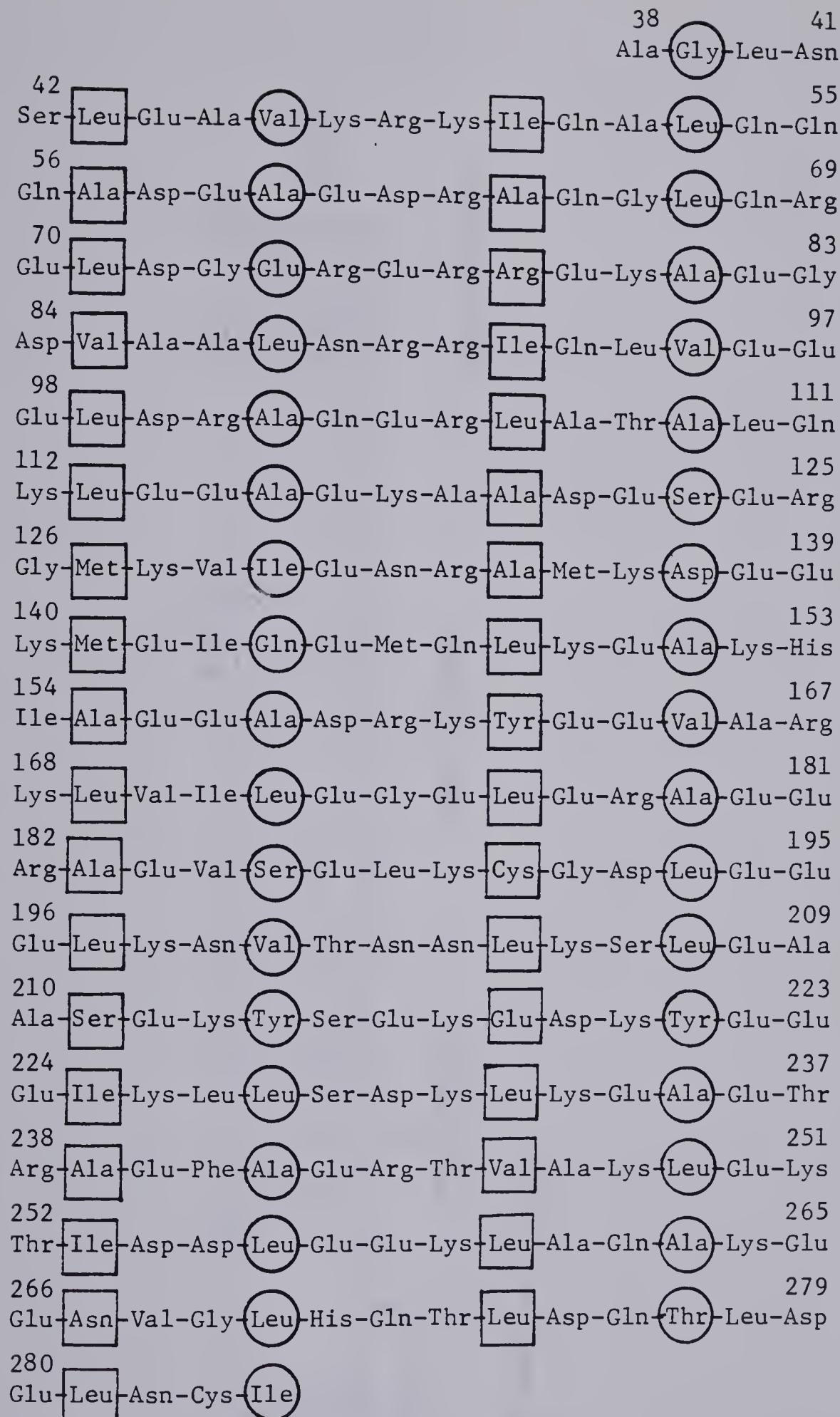


Fig. 33. Amino acid sequence of the major form (β) of platelet Tm. Non-polar residues occur in two series. Series I residues are squared; series II residues are circled. Numbering of the residues is based on the sequence of skeletal α Tm (Stone & Smillie, 1978).

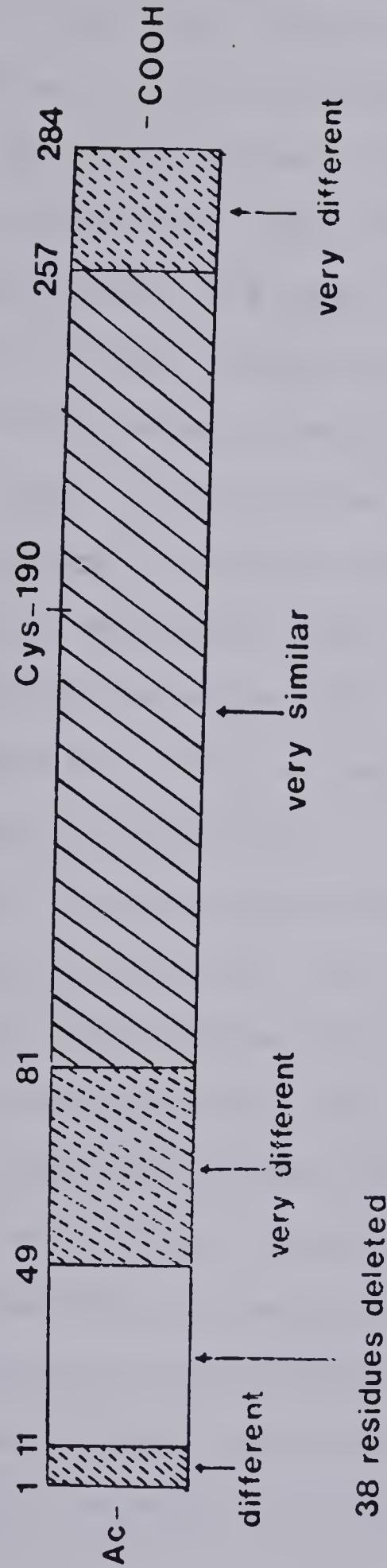


Fig. 34. Platelet Tm, here represented as a long rod, divided into regions based on a comparison of its amino acid sequence with that of skeletal α Tm. The 38 residue deletion can be placed anywhere within the region composed of residues 1 to 81.

formation of a coiled-coil structure are also found in the latter. Probably the most important of these is the repeating pattern of non-polar amino acids which extends throughout the length of the molecule. The non-polar residues are separated alternately by three or four positions and, since two turns of an α -helix contain almost seven amino acids, they tend to form a hydrophobic band down one side of the helix (Hodges et al., 1973). This can be seen more clearly if the sequence of Tm is divided into repeats of seven residues each. If each residue is labelled a to g (Fig. 35a) the repeating pattern of hydrophobic amino acids occupy the inner positions a and d. When two α -helical Tm chains come together interactions between the two non-polar series on each chain will form a hydrophobic core. This is thought to be the most important force stabilizing the coiled-coil.

Platelet Tm contains non-polar amino acids in the a and d positions throughout its length. Even those portions of the molecule which are very different from skeletal Tm have retained the hydrophobic repeat pattern. Only at positions 74 and 78, where a glutamic acid and arginine residue, respectively, fill core positions, does it appear possible that the coiled-coil structure may break down. At position 137 the presence of an aspartic acid residue in the hydrophobic core could lead to a local destabilization of the coiled-coil. The same residue in this position partially destabilizes the skeletal Tm structure (Pato, 1978)

The formation of salt bridges between charged residues in

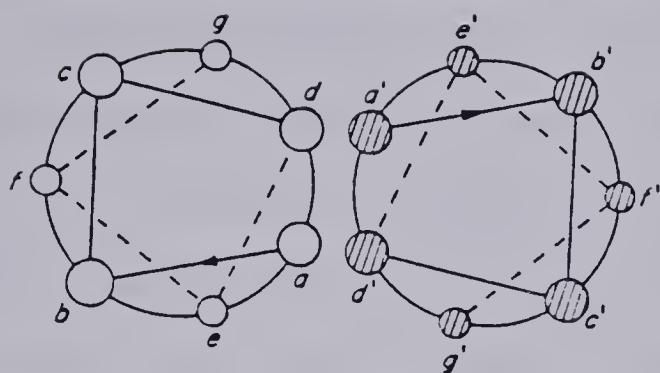
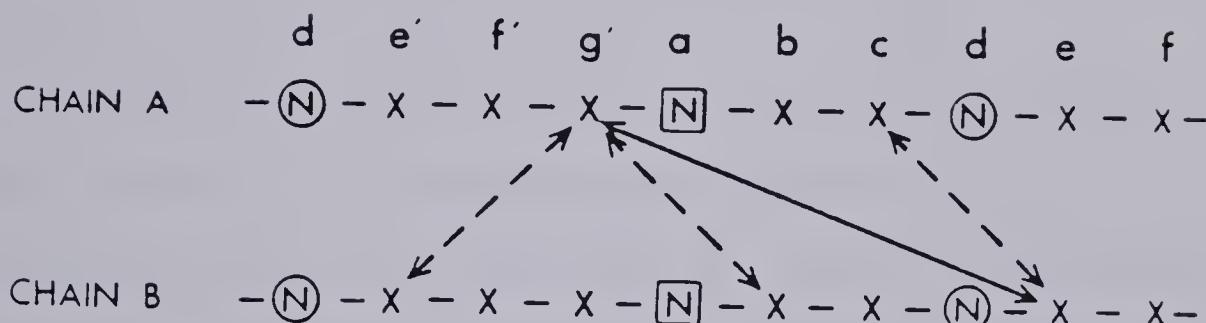
a.**b.**

Fig. 35a. End-on view looking from amino to carboxyl end of two α -helical chains of Tm in a coiled-coil structure. The non-polar residues in the a and d positions of one helix interlock with amino acids in the same positions of the other helix. [From McLachlan & Stewart, 1975]

Fig. 35b. Possible ionic interactions between heptapeptide sequences of two chains of Tm. The most favourable interactions are those between the residues occupying position e in one polypeptide chain with those occupying position g of the preceding heptapeptide (g') on the other polypeptide chain (indicated by solid line). [From Stone et al., 1974].

positions e of one helix and positions g of the second helix is thought to influence the stability of the coiled-coil structure (Stone et al., 1974; McLachlan & Stewart, 1975). In platelet T_m as in skeletal, a high proportion of basic residues are found in the g position and a high proportion of acidic residues in the e position (Table XV). From model building studies with skeletal T_m it was found that a residue occupying e would be most likely to interact with a side chain of the residue in position g of the preceding heptapeptide (g'). Other possible interactions (dotted lines in Fig. 35b) are sterically hindered by the presence of bulky hydrophobes occupying a and d (Stone et al., 1974).

To maximize favourable interactions between positively charged residues at g' and negatively charged groups at e the two chains of skeletal T_m must run parallel, rather than anti-parallel, to each other. Stone et al., (1974) and McLachlan and Stewart (1975) found that the greatest number of favourable interactions occurred when the two chains of T_m were in register rather than being staggered relative to each other. An exactly analogous situation occurs with platelet T_m and Table (XVI) indicates the correlation between the charges of amino acids at positions g' and e. There are 13 acidic residues compensated for by basic residues (including histidine) with like charges paired only twice when the chains are parallel and in register.

Since to form a coiled-coil structure both subunits must be in the α -helical conformation the tendency to form an α -helix should be high. Chou and Fasman (1974) have derived conformational

TABLE XV

Distribution of Acidic, Basic and Non-polar Residues in
the Repeating Heptapeptide Sequence of Platelet Tm and
Skeletal α Tm ()

Residue	Position in heptapeptide				
	a	d	e	g	b,c,f
Asp	0	1	1	1	12
Glu	1	1	18	7	27
Acidic	1 (1)	2 (1)	19 (23)	8 (12)	39 (44)
Lys	0	0	3	9	13
Arg	1	0	1	7	9
His	0	0	1	0	0
Basic	1 (2)	0 (0)	5 (4)	16 (19)	22 (28)
Ala	7	11	1	2	9
Leu	14	10	3	0	3
Tyr	1	2	0	0	0
Ile	4	2	0	1	2
Val	2	4	0	0	4
Met	2	0	0	0	2
Non-polar	30 (26)	29 (36)	4 (4)	3 (3)	20 (24)

TABLE XVI

Interactions Between Residues in the g' and e
Positions of Platelet Tm^a

		e position			
		Acid	Basic	Neutral	Total
g' position	Acid	1	2	5	8
	Basic	11	1	4	16
	Neutral	6	2	2	10
	Total	18	5	11	34

^a These figures are for platelet Tm chains parallel and in register.

parameters for each of the amino acids related to their likelihood of either forming an α -helix, β -sheet or random coil. When the sequences of skeletal and platelet T_m are analyzed in terms of the α -helix parameter the values obtained are always greater than one, indicating the presence of helix. Fig. 36 displays the result of averaging the α -helix parameter over stretches of 14 residues, plotted as a function of the peptide bond position, for both skeletal (Smillie et al., In Press) and platelet T_m.

The plot for skeletal α T_m displays a repeating periodic series of peaks and troughs, well developed at the amino terminus but becoming progressively less distinct towards the carboxyl terminal end of the molecule. Although it is not known to what extent the stability of the coiled-coil is dependent on this α -helix parameter, the wave like nature of the plot is consistent with much evidence suggesting that T_m is not a uniformly stable coiled-coil throughout its length, but has regions which are more flexible and mobile than others (see Smillie et al., In Press, and references therein). Pato and Smillie (1978) have shown that fragments from the carboxyl terminal half of skeletal T_m are less stable than those derived from the amino terminal half. This may in part result from the less developed periodicity and the lower average value of the α -helix parameter plot for the carboxyl terminal regions.

The smoothed α -helix parameter plot for platelet T_m diverges significantly from that of the skeletal protein near the two ends (Fig. 36). Overall, the changes make the plot for platelet

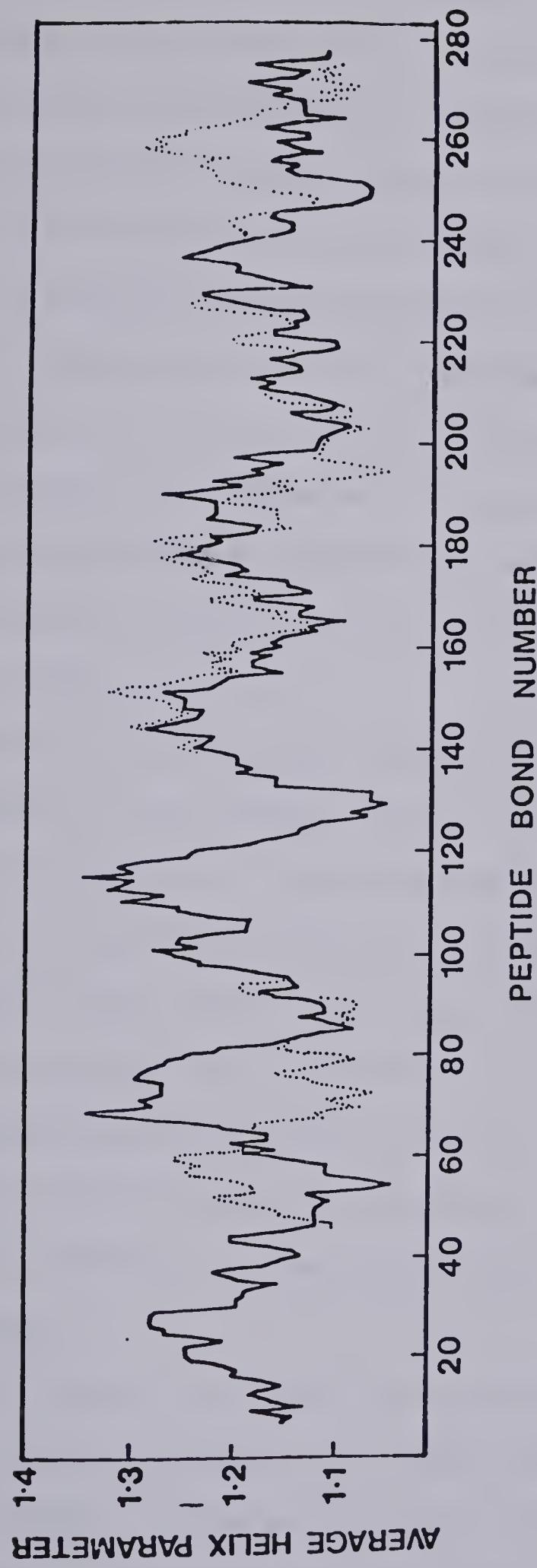


Fig. 36. Plot of the smoothed α -helix parameters for platelet (•••) and skeletal α (—) Tms. The α -helix parameters of individual amino acids have been averaged over stretches of 14 residues and plotted as a function of the amino acid at the center of each stretch.

T_m appear more uniform and less periodic than that of skeletal T_m. If fragments were prepared from platelet T_m it may be predicted from this plot that little difference in stability would be observed between the amino and carboxyl terminal halves.

This brief analysis of the important structural features of the muscle and non-muscle T_m sequences fails to reveal any obvious differences which would lead to a significant alteration in the conformational stability of the platelet protein when compared with that of skeletal. The conclusion drawn from the sequence comparison is that platelet T_m is an asymmetric, rod-like, α -helical coiled coil. A summary of the other experimental evidence fully supports this conclusion.

The M.Wt. of platelet T_m, measured in benign media by ultracentrifugation, is twice that measured under denaturing conditions (SDS gel electrophoresis) indicating that the native protein is a dimer. Gel filtration experiments in benign media indicated an asymmetric shape, with a Stokes radius only slightly smaller than that of skeletal T_m. The ability of the platelet molecule to aggregate into paracrystals again suggests a rod-like conformation and provides direct evidence for the length of the protein, which corresponds closely to the length expected for a completely coiled-coil structure.

The CD results confirm the high percentage of α -helix in the platelet protein's structure. The results are not of sufficient accuracy, however, to disclose whether or not a small section, perhaps 10-15%, of the structure is in a non-helical conformation.

The fact that the α -helical content did not increase when measured in 80% trifluoroethanol provides some evidence to suggest that platelet Tm is, under benign conditions, a complete coiled-coil.

The sequence of platelet Tm places all three tyrosine residues into hydrophobic core positions, as expected from the red shifted absorbance maximum observed for the protein. The intensity of the near UV CD spectrum, contributed mainly by tyrosine residues, suggests that the two subunits of the protein are aligned in register, which is in agreement with the analysis of the ionic interactions at the e and g positions. This important point needs to be confirmed by experiments such as those performed by Johnson and Smillie (1975), in which it was demonstrated that the cysteines of both unmodified and cross-linked skeletal Tm were capable of forming intramolecular disulfide bonds, a situation which could only occur if the two chains were parallel and in register.

Platelet Tm splits into two bands, termed α and β , when run on SDS urea or Laemmli gels, with the β form predominating. The sequence of platelet Tm (Fig.33) was performed on preparations composed largely (75%) of the β form, so that the amino acid substitutions contributed by the α form may not generally have been observed. It will be necessary to develop a method to separate α and β platelet Tm before the sequence of α can be determined and differences between the two forms revealed.

Based on the results of this section the question of the purity of the platelet Tm preparation can be assessed. SDS gel electrophoresis had previously demonstrated little contamination

with other proteins, a result supported by amino acid analyses, which indicated the absence of proline and yielded values for each amino acid nearly identical to those obtained by summation from the sequence. The A_{280}/A_{260} ratio rules out the presence of appreciable quantities of nucleic acid.

Only the $E_{280}^{1\%}$ value differentiates between platelet Tm prepared by method 1 and method 2, and, as well, indicates the possible presence of impurities. Based on tyrosine content and M.Wt., the $E_{280}^{1\%}$ value of platelet Tm should only be 58% that of skeletal Tm yet, depending on the method of preparation, it varies between 73 and 83%. The reason for this anomaly is not known. It may for the moment, as with the case of the anomalously high absorbance of Tn-C (Greaser & Gergely, 1973), have to be ascribed to some unknown chromophore, which may also be involved in the absorption from 290-340 nm.

The difference in $E_{280}^{1\%}$ measured for platelet Tm prepared by method 1 and method 2 presumably reflects a small difference in the purity of these preparations, a difference which was not reflected in their other properties. The majority of experiments described in this thesis were first performed with material isolated by method 1, then later repeated with material prepared by method 2, with no significant difference in their behaviour observed.

CHAPTER V

INTERACTION STUDIES WITH PLATELET TM

Skeletal muscle Tm has only a single known function, and that is to participate in the regulation of contraction. To perform this role it needs to display only a limited number of biological interactions, chief amongst which are the abilities to bind to the proteins actin (in its F form) and troponin. It is therefore natural to assume that the non-muscle Tms interact with actin microfilaments to, in some way, regulate non-muscle contractile events. Such a role for the non-muscle Tms is strongly supported by the results of immunofluorescence studies (described in Chapter I), which reveal that in vivo the protein is bound to actin bundles. However, direct evidence for the ability of non-muscle Tm to behave in a manner similar to skeletal Tm in the regulation of contraction is lacking.

Such evidence has been obtained in this study by investigating the interaction of platelet Tm with proteins of the skeletal muscle contractile system. The results obtained can be compared directly to those in the literature concerning skeletal Tm, revealing which basic functional properties have been retained by the platelet protein and which have been lost. These studies will provide a starting point for investigations of the much more difficult question of how the non-muscle Tms actually behave in vivo.

This chapter will begin with a discussion of the ability of platelet Tm to undergo the characteristic head to tail polymerization exhibited by muscle Tms, but the majority of the work presented will

be concerned with the interaction of platelet Tm with skeletal muscle actin and troponin. These interactions have been studied in two ways, by direct binding experiments, and, in the next chapter, by actin-myosin S-1 ATPase assays. The information derived from these two methods complement each other and can be correlated to develop a model depicting a possible means by which platelet Tm could be involved in the regulation of non-muscle contractile events.

A. HEAD TO TAIL POLYMERIZATION OF TM

A characteristic property of skeletal muscle Tm is its ability to aggregate head to tail at low ionic strengths, a phenomenon which results in a large increase in viscosity (Tsao et al., 1951; McCubbin et al., 1966). The interactions responsible for end to end polymerization have usually been interpreted in terms of the model put forward by McLachlan and Stewart (1975), in which an overlap of eight or nine residues occurs between two in-register coiled-coils, although recent x-ray crystallographic evidence indicates that the amino and carboxyl terminal ends of Tm molecules may intermesh to form small globular domains (Phillips et al., 1979).

McLachlan and Stewart postulate that the end to end overlap is stabilized by the formation of a hydrophobic core between the two broad faces of the coiled-coil. Experimental work demonstrates that the aggregation process is extremely sensitive to the chemical modification or enzymatic removal of those amino acids (important ones being Lys 7, Met 8, and Met 281) thought to be involved in the interior packing of the core (Johnson & Smillie, 1977).

In addition, the importance of electrostatic interactions

is evident from the fact that high ionic strength abolishes end to end aggregation. Fig. 37 shows the decrease in viscosity of a skeletal α Tm solution as the salt concentration is raised.

It should be mentioned that skeletal Tm, if dialyzed overnight against a buffer lacking reducing agent, or if left to stand for extended periods after dialysis against a buffer containing reducing agent, gave quite low viscosities, even at low ionic strength. This was presumably a result of oxidation, of either Cys 190 to a disulfide bond, or of Met 8 or 281 to a methionine sulfoxide. For this reason 2 mM mercaptoethanol was included in all buffers, and viscosities were recorded within the first one or two hours following removal of the Tm solution from the dialysis

Despite these precautions platelet Tm displayed only a small increase in viscosity as the ionic strength was lowered (Fig. 37), indicating a decreased ability, relative to skeletal Tm, for end to end polymerization. Further evidence for a decreased ability of the platelet Tm to aggregate was obtained by sedimentation equilibrium measurements. Preliminary experiments, in 0.1M KCl, 10 mM Tris, pH 8.0, suggested that while skeletal α Tm was present largely as a mixture of dimers and trimers (M.Wts. of 140,000 to 160,000 daltons), platelet Tm was still mainly in the monomeric or dimeric form (M.Wts. of 50,000 to 90,000 daltons).

B. INTERACTION OF PLATELET TM WITH TROPONIN

A large increase in viscosity occurs when skeletal muscle troponin is added to a solution containing skeletal Tm, indicating

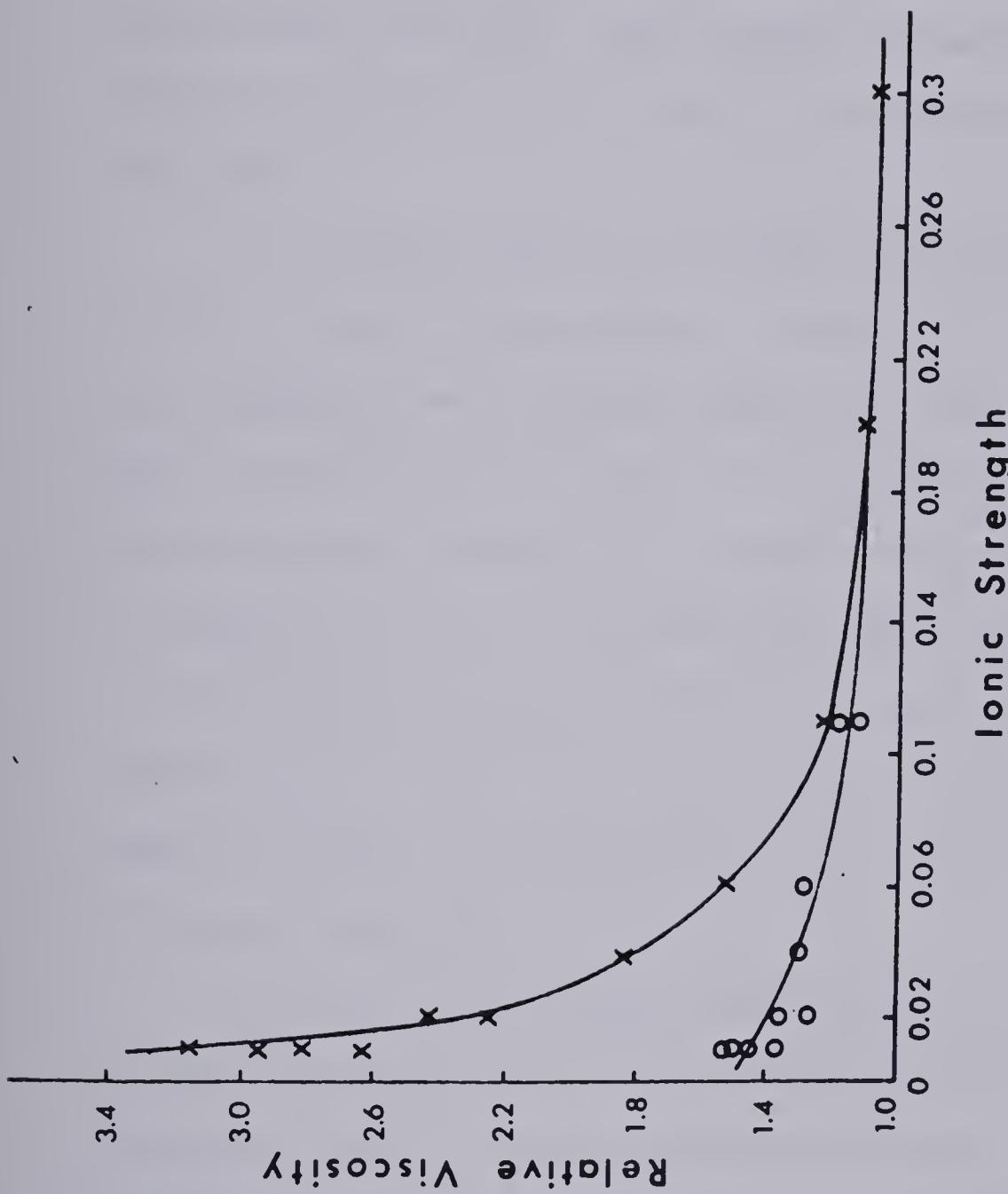


Fig. 37. Relative viscosity versus ionic strength relationship for skeletal α Tm (x) and platelet Tm (o). Proteins were dialyzed overnight against buffer containing 2 mM mercaptoethanol, 10 mM cacodylate, pH 7.0, with varying amounts of NaCl. The Tm concentration was 2 mg/ml.

an interaction between these two proteins (Ebashi & Kodema, 1965; Sugita et al., 1969). Even in a buffer of relatively high ionic strength (0.3M KCl), where skeletal Tm alone exhibits little viscosity, the addition of troponin is able to induce head to tail polymerization (Fig. 38). When troponin was added to a solution containing platelet Tm no increase in viscosity could be detected (Fig. 38).

The largest subunit of troponin, the Tm binding component, or Tn-T, is wholly responsible for the ability of troponin to increase the aggregation of Tm (Yamamoto & Maruyama, 1973; Jackson et al., 1975). Tn-T could not be used in these experiments as it is insoluble at low ionic strength, so a soluble fragment (CB-1) corresponding to residues 1 to 151 of Tn-T, and which shares with Tn-T the ability to bind to and enhance the viscosity of skeletal Tm, was used (Jackson et al., 1975). CB-1 was, however, like whole troponin, unable to produce a detectable rise in the viscosity of a platelet Tm solution (Fig. 39).

The possibility that platelet Tm was binding to troponin, but that this interaction was not leading to increased end to end aggregation, led to further experiments involving affinity chromatography columns (prepared by Dr. M. Pato), to which had been immobilized either troponin, Tn-T, or CB-1 (Pato & Smillie, 1978). Skeletal α Tm remained bound to all three types of affinity columns until a salt concentration of 0.2M was reached, while platelet Tm eluted at KCl concentrations ranging from 0.04 to 0.08M. Other coiled-coil polypeptides, such as myosin S-2 and light meromyosin,

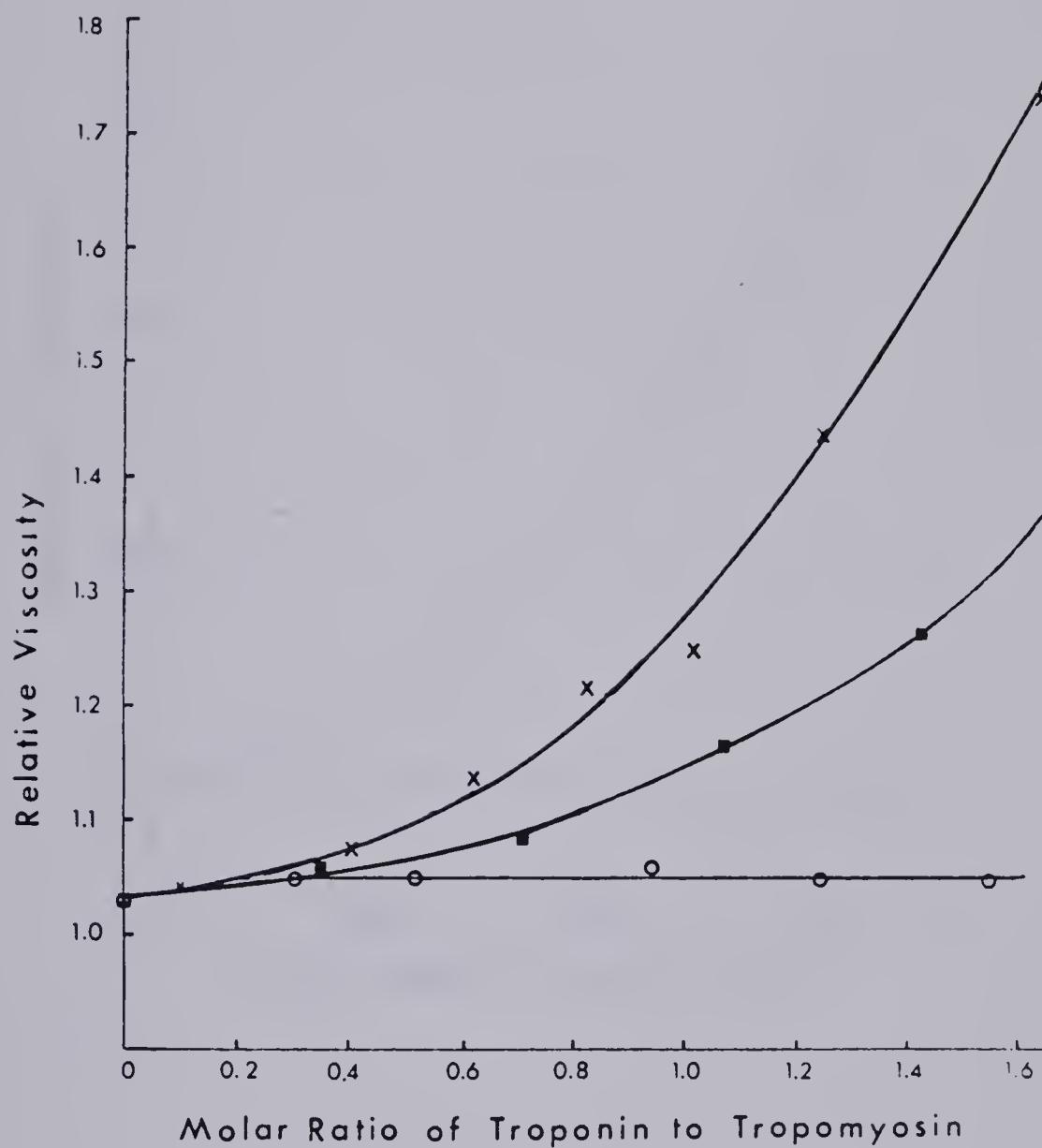


Fig. 38. Effect of the addition of skeletal troponin on the viscosity of α skeletal Tm (x , ■) and platelet Tm (\circ) solutions. Experiments were performed in 2 mM mercaptoethanol, 10 mM cacodylate buffer, pH 7.0, with either 0.1M (x, \circ) or 0.3M (■) NaCl. The Tm concentration was always 0.5 mg/ml.

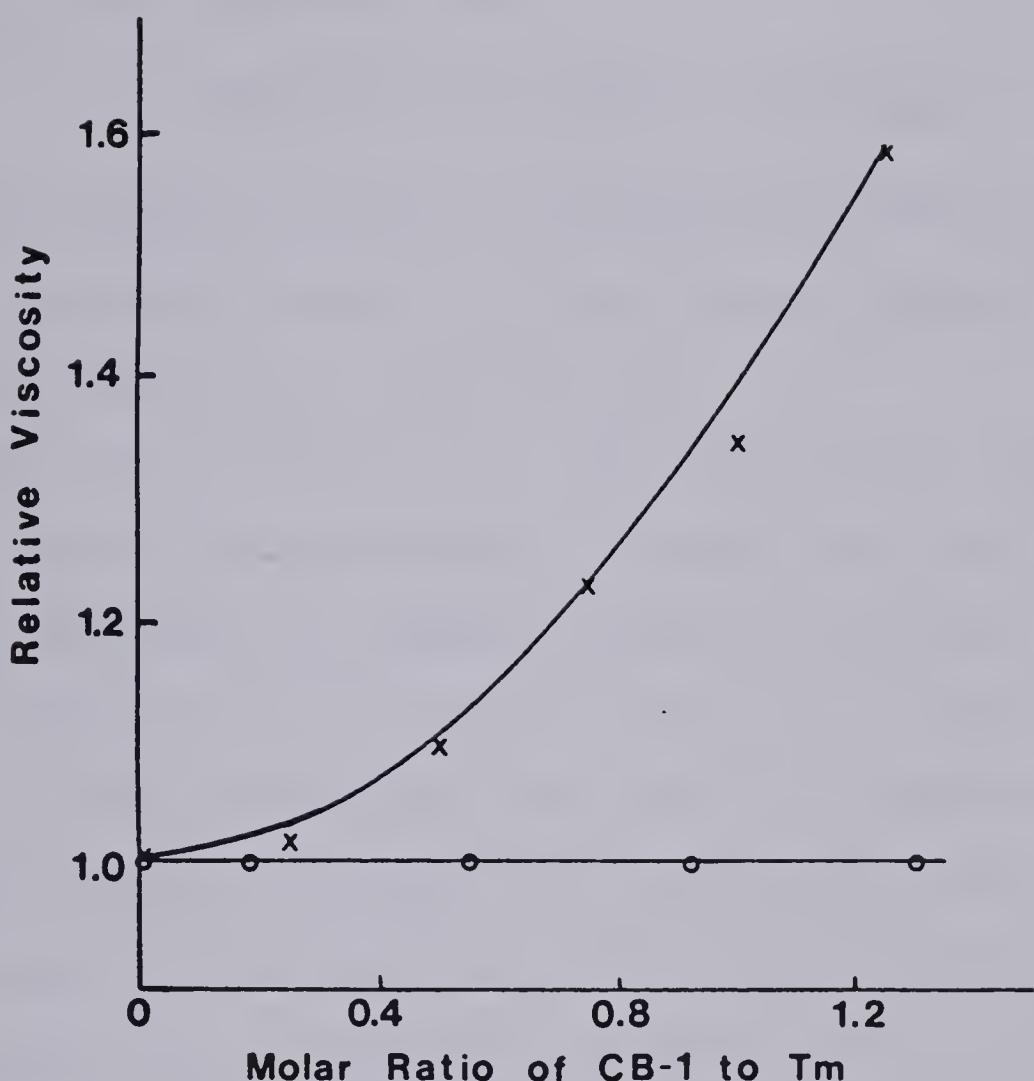


Fig. 39. Effect of the addition of CB-1 on the viscosity of a skeletal Tm (x) and platelet Tm (o) solutions. Experiments were performed in 0.1M NaCl, 5 mM mercaptoethanol, 20 mM cacodylate buffer, pH 7.0. The Tm concentration was 0.5 mg/ml, and the CB-1 concentration was determined by amino acid analysis.

which would interact with the troponin affinity columns in a strictly non-specific manner, eluted under conditions similar to those of platelet Tm (Pato & Smillie, 1978).

C. INTERACTION OF PLATELET TM WITH ACTIN

The binding of platelet or skeletal Tm to actin was determined by measuring the amount of protein which co-sedimented with the F-actin pellet during centrifugation.

For most experiments, the amount of Tm present in the F-actin pellet, taken as being equivalent to the amount bound, was quantitated by photometrically scanning SDS gels of the pellets. This method, which gave accurate values for the ratio of staining intensities of the two proteins, was well suited for following increases in binding; however, the conversion of the staining intensities back to values representing actual protein concentration did not give reliable results. Standard curves depicting the relationship between staining intensity and protein concentration could not be obtained with the desired reproducibility. For this reason, attempts were made to obtain quantitative values for the amount of platelet Tm bound to actin by using ^{125}I -labelled platelet Tm.

1. Effects of Solution Conditions

Quite a number of factors are capable of affecting the binding of skeletal Tm to actin. Some, including temperature, pH, and the presence or absence of urea act by altering the α -helical content of Tm; a decrease in binding strength can be correlated with a less perfect coiled-coil structure (Tanaka, 1972). These variables

have not been tested for their ability to perturb the binding of platelet Tm to actin, but it is expected their effects would parallel those found for skeletal Tm.

By far the most dramatic alteration in the binding of Tm to actin is caused by divalent cations (Maruyama, 1964; Tanaka, 1972; Eaton et al., 1975; Wegner, 1979; Yang et al., 1979a). At low salt concentrations skeletal Tm is unable to bind to actin in the absence of divalent cations, although upon addition of 2 to 3 mM free Mg⁺⁺ stoichiometric binding is induced. The binding of both skeletal and platelet Tm to actin as a function of the Mg⁺⁺ concentration is shown in Fig. 40. The results for skeletal Tm are similar to those recently published by Yang et al. (1979) and Wegner (1979), but for platelet Tm the threshold level of Mg⁺⁺, where binding begins, and the optimal level, where binding is complete, are much higher.

At 8 mM free Mg⁺⁺ the binding of platelet Tm to skeletal actin is virtually stoichiometric (Fig 41). Substitution of a non-muscle actin, isolated from platelets, for skeletal actin, did not significantly affect the binding ability of the platelet Tm (Fig. 40).

It is not clear how Mg⁺⁺ enhances the ability of Tm to bind to actin. Mg⁺⁺ may form salt bridges between negative groups on the two proteins, or, as Tanaka (1972) suggests, it may stabilize the F-actin structure, making it more rigid.

The ionic strength, to a limited extent, is involved in modifying the actin-skeletal Tm interaction. In the absence of divalent cations binding between the two proteins, nonexistent at first, increases as KCl is added, reaching a maximum amount at a

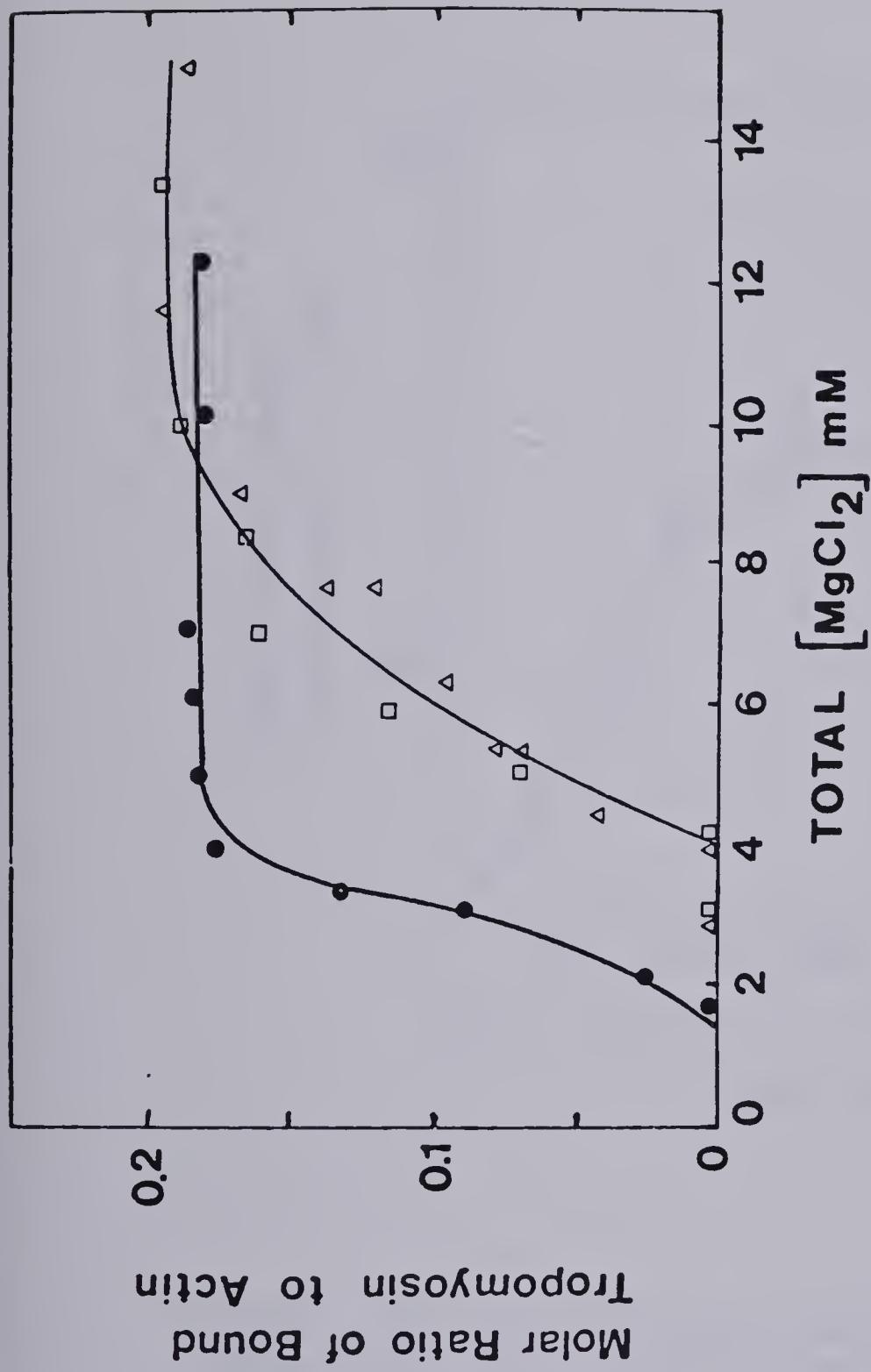


Fig. 40. Effect of Mg^{++} concentration on the binding of skeletal α Tm (●) and platelet Tm (□,△) to actin. Each centrifuge tube contained 0.42 mg actin and either 0.21 mg skeletal Tm or 0.17 mg platelet Tm (giving a molar ratio to actin of 2:7) in 1.5 ml of buffer (30 mM KCl, 2 mM DTT, 2 mM EGTA, 2 mM Tris, pH 7.8 with varying concentrations of $MgCl_2$). Due to the presence of 2 mM ATP the free Mg^{++} concentration remains zero until 2 mM $MgCl_2$ has been added. Assays marked (●,□) were performed with skeletal actin, while those marked (△) used platelet actin. Values were obtained by scanning SDS gels of the actin pellets.

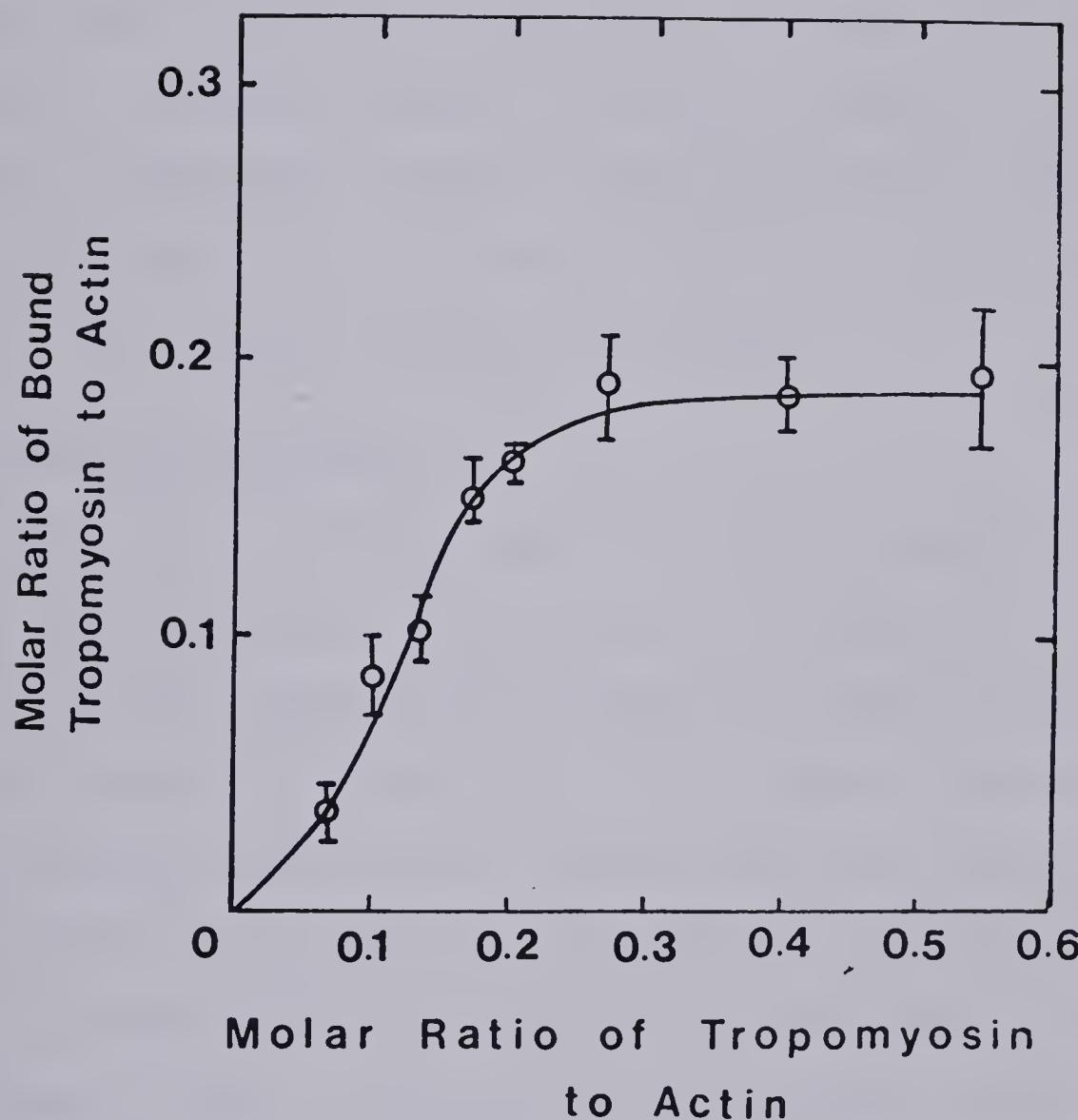


Fig. 41. The binding of platelet Tm to skeletal actin at a MgCl_2 concentration of 10 mM (8 mM free Mg^{++}), otherwise the buffer is as described in the legend to Fig. 40. Each assay contained 0.38 mg actin in a 1.5 ml volume. The bars indicate the highest and lowest molar ratios obtained for each point for four separate runs on SDS gels. Values determined by gel scanning.

salt concentration of 0.1M (Tanaka, 1972; Eaton et al., 1975).

At a free Mg^{++} concentration of 2.5 mM, the weak binding which occurs with platelet Tm at low salt can be enhanced by the addition of KCl, until, as with skeletal Tm, an optimal salt concentration (0.1 to 0.12M) is reached (Fig. 42). Even under these optimal salt conditions, however, the amount of platelet Tm bound to actin is well below that which is observed at higher levels of Mg^{++} . It appears that at low levels of Mg^{++} , KCl is not an effective inducer of the binding of platelet Tm to actin.

2. Effects of Other Proteins

Not only the solution conditions, but the presence of certain other proteins are able to alter the binding of Tm to actin. Eaton et al. (1975) showed that the affinity of skeletal Tm for actin could be increased by the addition of Tn-I. Similar results were found with platelet Tm (Fig. 43). Under conditions (3 mM free Mg^{++}) where platelet Tm by itself bound only partially to actin, the addition of increasing amounts of Tn-I progressively raised the amount of platelet Tm bound to actin. Preliminary evidence indicates that troponin enhances the binding of platelet Tm to actin in much the same manner as Tn-I alone does.

Myosin heads, when bound strongly to actin in rigor complexes (due to the absence of ATP), have been shown to increase the binding affinity of skeletal Tm for actin (Eaton, 1976). The same phenomenon, using skeletal myosin S-1, was observed with platelet Tm (Fig. 44). The gel scanning method was used to determine the molar ratios of platelet Tm to actin for this experiment, which produced difficulties

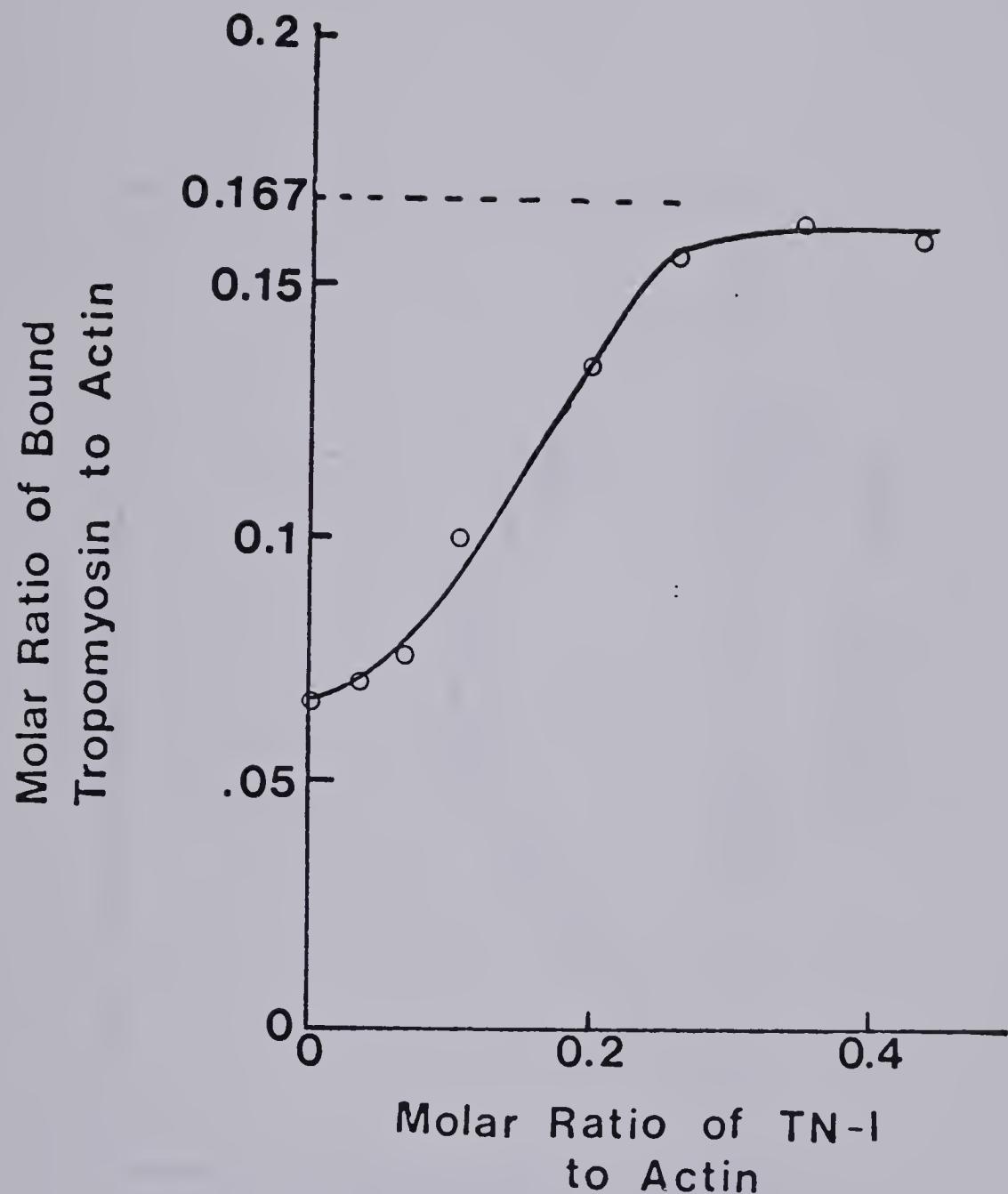


Fig. 43. The effect of increasing amounts of Tn-I on the binding of platelet Tm to skeletal muscle actin. Each centrifuge tube contained 0.20 mg/ml actin and 0.086 mg/ml of ^{125}I -labelled platelet Tm (a 0.31 molar ratio to actin) in 1.0 ml of buffer consisting of 30 mM KCl, 2 mM DTT, 3 mM MgCl_2 , 2 mM Tris, pH 7.8. Three 100 μl samples were taken from each tube before and after centrifugation to determine radioactivity.

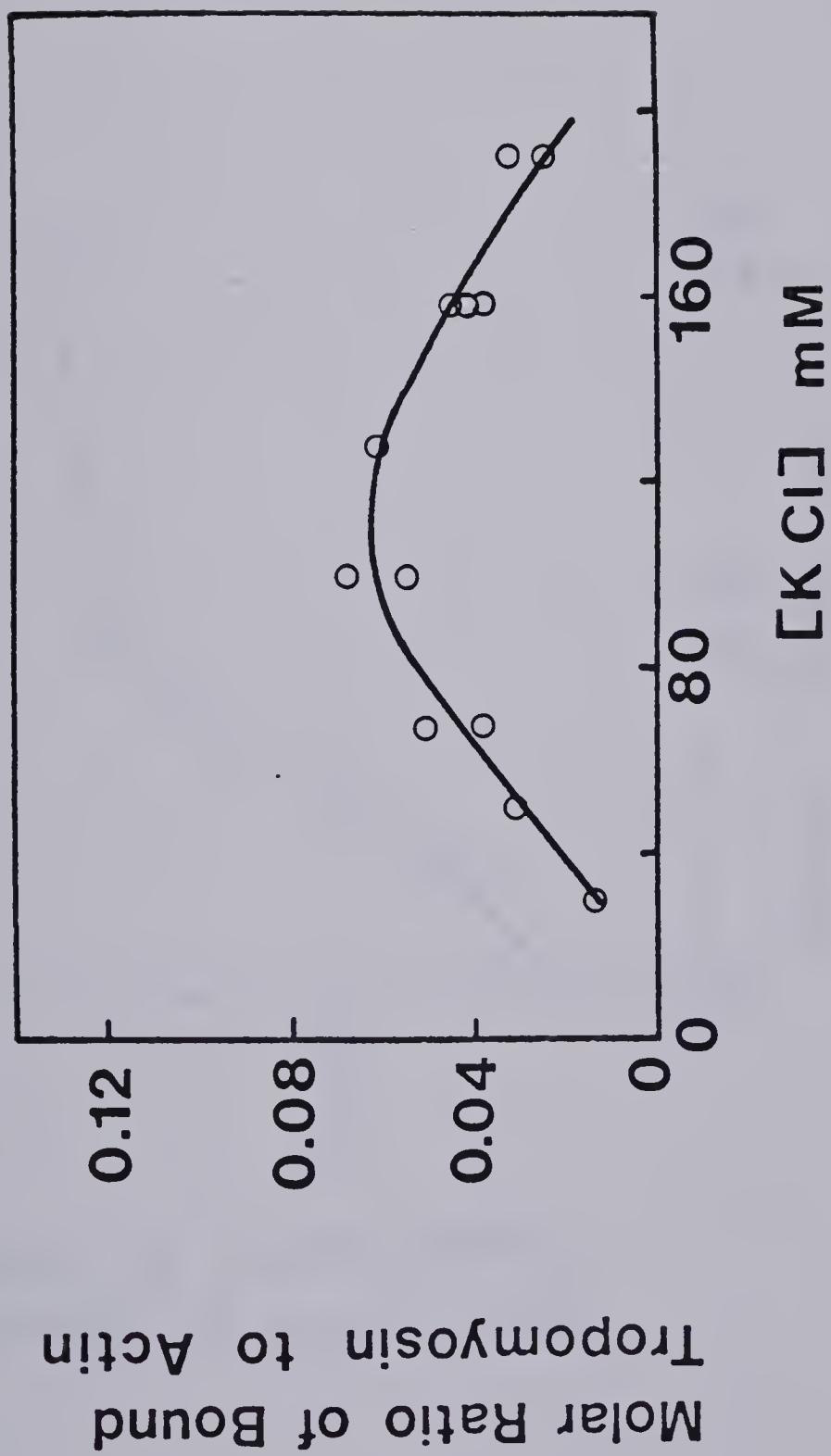


Fig. 42. Effect of the KCl concentration on the binding of platelet Tm to skeletal actin. The platelet Tm concentration was 0.15 mg/ml while actin was 0.35 mg/ml. Buffer contained 2 mM DTT, 1.0 mM EGTA, 2 mM ATP, 4.5 mM MgCl₂, 2 mM Tris, pH 7.8, with varying concentrations of KCl.

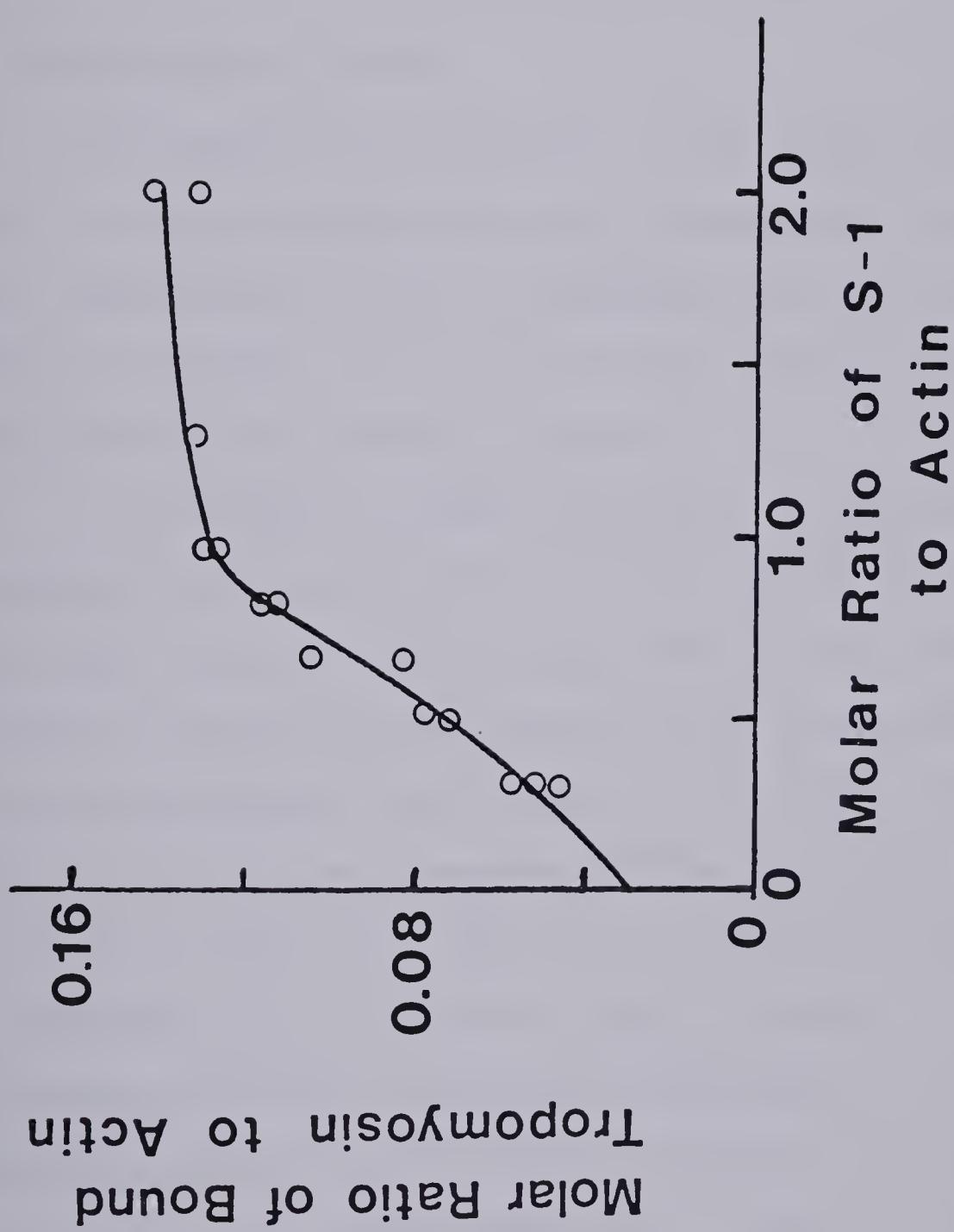


Fig. 44. The effect of increasing amounts of S-1 on the binding of platelet Tm to skeletal actin. The assay contained 0.35 mg/ml actin, 0.112 mg/ml platelet Tm (a 0.23 molar ratio to actin) in 1.4 ml of buffer as described in the legend to Fig. 43. Molar ratios were determined by scanning gels.

since the A-1 light chain peak and platelet T_m peak partially overlapped. Corrections made for this overlap may have resulted in inaccurate molar ratios, since the curve levels off at values lower than expected (compare Fig. 44 with Fig. 40 and 41). Alternatively, it may be that the binding of so many myosin heads to actin sterically hinders the binding of some platelet T_m molecules.

3. Stoichiometry of Binding

The binding studies described in this chapter have shown that there is a definite limit to the amount of platelet T_m which is able to associate with actin. A quantitative estimate of this limit would be of interest, as it would define the number of actin binding sites on each of the platelet T_m molecules.

In some studies the amount of platelet T_m bound to actin was determined by scanning SDS gels of the actin pellet after centrifugation (Fig. 40 and 41). These curves level off at a molar ratio of about one platelet T_m molecule to five F-actin monomers, but they have not been corrected for differences in the ability of the two proteins to bind the dye (Coomassie Brilliant Blue R-250). Preparation of a standard curve indicated that platelet T_m and skeletal α T_m bound this dye to an identical extent, therefore the knowledge that skeletal T_m binds to seven actins can be used to correct the results for platelet T_m. The binding of skeletal α T_m levels off at a molar ratio slightly below that of platelet T_m (Fig. 40), suggesting that the true platelet T_m to actin molar ratio is somewhat greater than one to seven.

An attempt was made to determine the binding ratio of platelet Tm to actin in a more direct manner through use of Tm iodinated with ^{125}I . The radioactivity in a solution containing actin and $^{125}\text{I}^-$ -platelet Tm was measured before and after centrifugation, and the difference was taken to represent the amount of Tm bound to the actin. The amount of platelet Tm sedimenting in the absence of actin was not significant.

Using this method, it was found that the saturation limit of platelet Tm binding to actin (when binding was induced by the addition of Tn-I) was close to a one to six molar ratio (Fig. 43). Variable results were obtained when a number of separate experiments were performed at high concentrations of free Mg^{++} (Table XVII). The results rule out the binding of platelet Tm to actin in a one to five molar ratio, but are not of sufficient accuracy to distinguish between a one to six or one to seven molar ratio. Most of the values obtained lie somewhere between these two ratios.

The shorter length of platelet Tm makes it likely that this protein is only capable of binding to six actins. Perhaps the inability of platelet Tm to aggregate head to tail results in some F-actin monomers remaining uncovered between adjacent Tms, lowering the binding ratio below one to six. Further experiments will have to be performed to pinpoint the exact number of actin binding sites on platelet Tm.

TABLE XVII

Determination of the Amount of ^{125}I -Labelled
Platelet Tm Bound to Actin^a

^{125}I -Platelet Tm Added to the Assay	<u>Molar Ratio to Actin^b</u>	^{125}I -Platelet Tm Bound
0.36		0.151
		0.156
		0.180
0.52		0.158
		0.159
0.60		0.160
0.645		0.143
		0.151
		0.176
0.91		0.167
1.03		0.180
Average		0.162
Standard Deviation		0.012

a) experiments performed in 30 mM KCl, 7 mM MgCl₂, 2 mM DTT,
0.1 mM EGTA, 10 mM Tris, pH 7.6

b) a 1 to six molar ratio equals 0.167, a 1 to seven ratio 0.142

D. DISCUSSION

The interaction studies described in this chapter reveal a number of important differences between the properties of skeletal α Tm and platelet Tm. Platelet Tm has a lower affinity for F-actin than skeletal Tm, and appears to have lost the abilities to bind Tn-T and polymerize in a head to tail manner. Platelet Tm does, however, appear able to interact with Tn-I in a manner comparable to that of skeletal Tm. This discussion will attempt to provide explanations for these observations based on an analysis of the structural features of the platelet and skeletal molecules.

The virtual loss of the ability of platelet Tm to polymerize head to tail can probably be attributed to changes in the amino and carboxyl terminal sequences of this protein, when compared to skeletal α Tm (Fig. 45). Although replacements at the carboxyl terminus of the platelet protein can be considered fairly conservative (Leu 281 should be able to replace Met 281 in the same manner as Ile 281 does in skeletal β Tm (Johnson & Smillie, 1977)) the amino terminus has lost many residues which, according to the model of McLachlan and Stewart (1975), are most important in stabilizing the end to end overlap. The bulky Met 8 found in skeletal Tm is replaced by an alanine residue, thus opening up the hydrophobic core to solvent, while Asp 2, Lys 5, and Lys 6 of the skeletal protein, all involved in salt bridges, are replaced by neutral amino acids in platelet Tm.

It is thought that the ability of muscle Tm to aggregate head to tail plays a vital role in the orderly alignment of the Tm-troponin complex on the actin filament, which ensures that no

Amino Terminal Sequences

Platelet Tm Ac-Ala-Gly-Leu-Asn-Ser-Leu-Glu-Ala-

Skeletal α Tm Ac-Met-Asp-Ala-Ile-Lys-Lys-Lys-Met-

Carboxyl Terminal Sequences

Platelet Tm -Leu-Asp-Glu-Leu-Asn-Cys-Ile-COOH

Skeletal α Tm -Leu-Asn-Asp-Met-Thr-Ser-Ile-COOH

Fig. 45. A comparison of the amino and carboxyl terminal sequences of skeletal α and platelet Tm.

F-actin monomers are left unregulated. Tawada et al. (1975) have shown that the ability of skeletal Tm molecules on the thin filament to interact with each other results in a cooperativity which allows inhibition of the actin-myosin interaction to be released over a narrower than expected range of Ca^{++} concentration. It is possible then, that platelet Tm, by reason of its inability to form end to end associations, may be generally less effective than skeletal Tm in regulating contraction. However, the lack of a detailed study on the ability of non-polymerizing skeletal Tm (which can be formed by means of digestion with carboxypeptidase (Tawada et al., 1975; Johnson & Smillie, 1977)) to dictate the periodicity of the troponin complex on the thin filament, and to regulate contraction, makes such a conclusion highly speculative.

The results obtained by affinity chromatography, and by viscosity measurements, demonstrated that platelet Tm bound to troponin much more weakly than did skeletal Tm. The result was unexpected since at the time the binding site for Tn-T on skeletal Tm was postulated to be in the region of residues 197 to 217 (for a summary of the evidence on which this hypothesis is based see McLachlan & Stewart, 1976b; Pato, 1978). Comparison of the sequences shows that in this area platelet Tm differs from both skeletal α and β Tm in only four positions (Fig. 46).

At only a single one of these positions (residue 216), in which a glutamine in α and a threonine in β are changed to a glutamic acid residue in platelet Tm, can the alteration be considered as of significance in possibly altering the interaction of platelet Tm with

Muscle α Tm	183	184	185	186	187	188	189	190	191	192	193	194
	Glu -	Leu -	Leu -	Ser -	Glu -	Gly -	Lys -	Cys -	Ala -	Glu -	Leu -	Glu
Muscle β Tm	—	—	—	Val -	Ala	—	Ser	—	—	Gly -	Asp	—
	—	—	—	Val	—	—	Leu	—	—	Gly -	Asp	—
Platelet Tm	—	—	—	—	—	—	—	—	—	—	—	—
	195	196	197	198	199	200	201	202	203	204	205	206
Muscle α Tm	Glu -	Glu -	Leu -	Lys -	Thr -	Val -	Thr	Asn -	Asn -	Leu -	Lys -	Ser
Muscle β Tm	—	—	—	—	—	—	—	—	—	—	—	—
Platelet Tm	—	—	—	—	—	—	—	—	—	—	—	—
	207	208	209	210	211	212	213	214	215	216	217	218
Muscle α Tm	Leu -	Glu -	Ala -	Gln -	Ala -	Glu -	Lys -	Tyr -	Ser -	Gln -	Lys -	Glu
Muscle β Tm	—	—	—	—	—	—	Asp	—	—	—	Thr	—
Platelet Tm	—	—	—	—	—	Ala -	Ser	—	—	—	Glu	—
	219	220	221	222	223	224	225	226	227	228	229	230
Muscle α Tm	Asp -	Lys -	Tyr -	Glu -	Glu -	Glu -	Ile -	Lys -	Val -	Leu -	Ser -	Asp
Muscle β Tm	—	—	—	—	—	—	—	—	Leu	—	Glu	—
Platelet Tm	—	—	—	—	—	—	—	—	Leu	—	—	—
	231	232	233	234	235	236	237	238	—	—	—	—
Muscle α Tm	Lys -	Leu -	Lys -	Glu -	Ala	Glu -	Thr	Arg	—	—	—	—
Muscle β Tm	—	—	—	—	—	—	—	—	—	—	—	—
Platelet Tm	—	—	—	—	—	—	—	—	—	—	—	—

Fig. 46. Comparison of amino acid sequences of rabbit skeletal muscle α and β Tms and platelet Tm. Numbering scheme is for the skeletal Tms (Stone & Smillie, 1978). Residues which are identical to those of skeletal α Tm are represented by (—). The hypothetical Tn-T binding region is shown boxed.

Tn-T. It is unlikely that this one charge change could dramatically alter the Tn-T binding properties of platelet Tm, although of course such a possibility exists.

These observations therefore raise a question as to the validity of the assignment of the Tn-T binding site on skeletal Tm to a position one-third of the distance from the carboxyl terminus of the molecule. They suggest that consideration must be given to the possibility that the binding site is located closer to the carboxyl terminal end of the molecule, perhaps even involving the head to tail overlap region of Tm aggregates.

Recent work in our lab (A. Mak, M. Pato & L.B. Smillie) has in fact produced convincing evidence to indicate that Tn-T does bind near the carboxyl terminus of skeletal Tm, in a region probably consisting of residues 260 to 284. In addition, Ohtsuki (1979), using antibodies to an amino terminal fragment of Tn-T (corresponding closely to CB-1), has demonstrated that this fragment binds to the thin filament in a position corresponding to the end to end overlap regions of skeletal Tm.

Since these are the regions most markedly altered in platelet Tm (Fig. 34), these recent results are consistent both with the primary structural data, as well as with the different interaction properties of platelet Tm with troponin. In addition, they provide an explanation for the ability of troponin to increase the head to tail polymerization of skeletal Tm.

The results in this chapter show that platelet Tm binds specifically to actin. By this it is meant that the amount of platelet

Tm which binds to actin, under all conditions tested, reaches a limiting value, most likely one platelet Tm to six F-actin monomers, which cannot be exceeded, since at this point the binding sites for platelet Tm on actin have been fully occupied.

Platelet Tm does not bind to actin with as high an affinity as skeletal Tm. This statement is justified primarily by the experiment shown in Fig. 40, since it has been shown that the binding constant of skeletal Tm to actin increases as the Mg^{++} concentration is raised (Wegner, 1979; Yang et al., 1979b). In order for platelet Tm to bind to actin in amounts similar to those of skeletal Tm the Mg^{++} content of the buffer must be increased by 2 to 3 mM (Fig. 40). Wegner (1979) provides data to indicate that a 2 mM increase in Mg^{++} produces a four- to five-fold increase in the affinity of skeletal Tm for actin; this then can be taken as a rough estimate of the extent to which the binding affinity of platelet Tm has decreased.

The major cause of the weakened binding is no doubt a result of the shorter length of the platelet Tm molecule; it has the ability to bind to only six actin monomers while skeletal Tm interacts with seven.

Whether any of the other changes in the amino acid sequence of platelet Tm may affect actin binding is open to conjecture. The actual residues of skeletal Tm involved in important interactions with actin are not clearly defined, although analysis of the protein's sequence has revealed that there is a quasi-equivalent arrangement of acidic residues, and outer non-polar residues (those in positions b, c, and f) repeated fourteen times along the length of the mol-

ecule (Stone et al., 1974; Parry, 1975; McLachlan & Stewart, 1976a). The length of each repeat, considered to represent one actin binding site, is about 19.6 residues, equal to the axial distance of about 2.9 nm between F-actin monomers.

In the same manner, platelet T_m can be divided up into twelve zones (Fig. 47). As in skeletal T_m, the zones can be further divided into two sets, labelled α and β , which alternate throughout the sequence (McLachlan & Stewart, 1976a). Only one of these sets is thought to bind strongly to actin at any one time. The other set would come into play if T_m altered its position on the actin filament, as is thought to happen when Ca⁺⁺ binds to troponin. That is, one set would bind to actin when T_m was in the inhibitory position, while the other set would bind when T_m was in the activated position, a situation which requires each actin monomer to have two similar actin binding sites 2.9 nm apart.

Each of the α and β bands is divided into a negative zone, a positive zone, and then another negative zone, based on net charge (Fig. 47). The outer non-polar residues are clustered into a distinct region of their own. There is little experimental evidence available by which to judge the relative contributions of each of the zones towards the formation of an actin binding site. Acidic residues probably play a major role in light of the remarkable effect of Mg⁺⁺ on binding affinity.

A comparison of Fig. 47 with the analogous figure for skeletal α T_m (McLachlan & Stewart, 1976a) reveals that all the acidic and outer non-polar residues are highly conserved in platelet T_m from

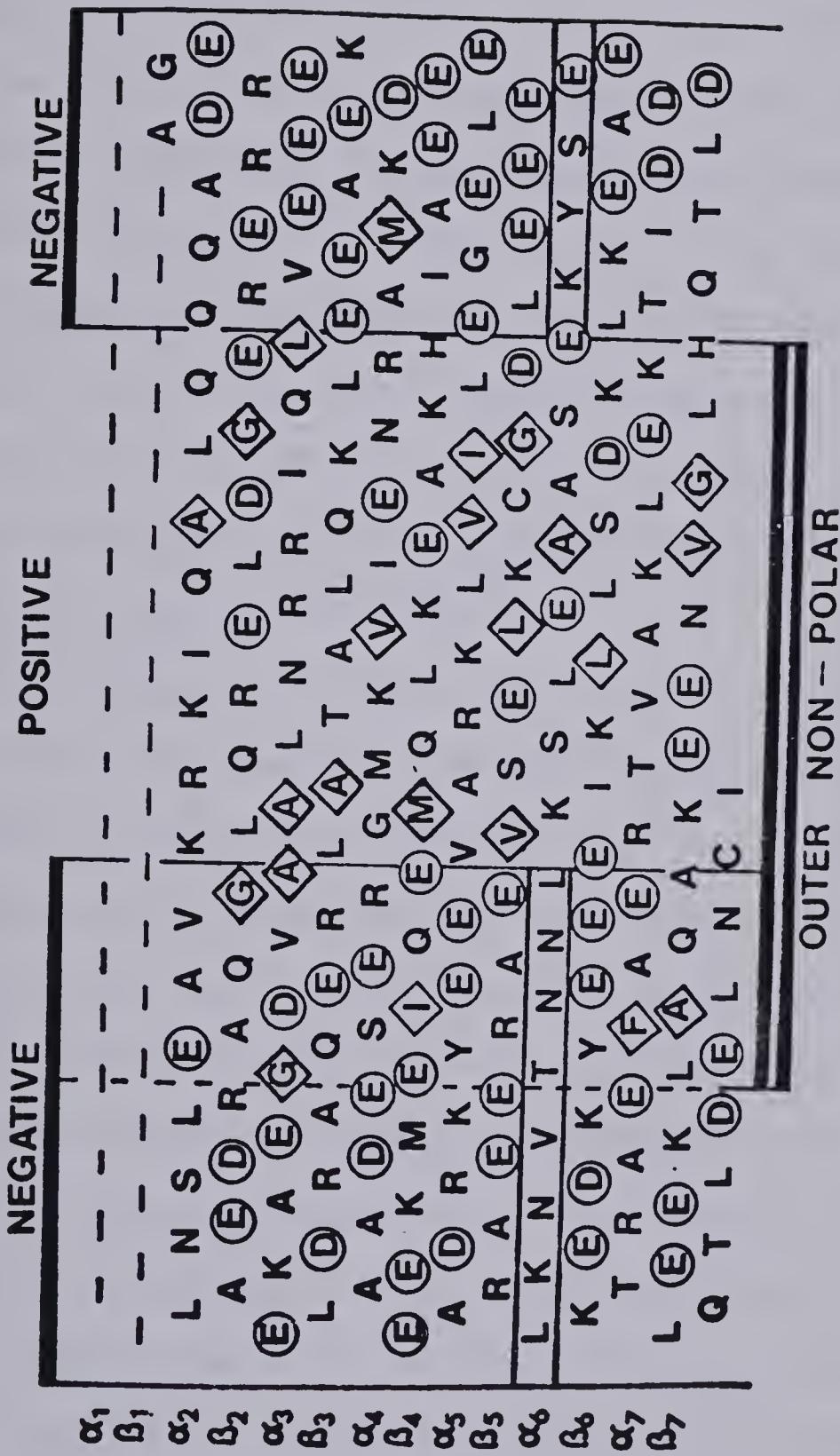


Fig. 47. The amino acid sequence of Platelet Tm in one letter code drawn in a 19 2/3 residue repeat pattern. The dashes (-) indicate the two repeats which have been deleted from the Platelet Tm sequence, but are present in skeletal Tm. Negative charges are ringed (0), outer non-polar residues in positions b, c and f are marked (\diamond), and the boxed residues indicate the region corresponding to the proposed Tn-T binding site on skeletal Tm. The zones marked negative and positive indicate the regions where amino acids with these charges predominate. The region where outer non-polar residues cluster is also indicated. The 19 2/3 residue repeats are divided into two alternating sets, α and β , which may interact with actin independently.

band α_3 through to, and including, band α_7 . In band β_7 , however, the negative and positive zones, well defined in skeletal Tm, have been greatly weakened in platelet Tm. Three negative charges have been lost from, and one positive charge added to, the negative zone, while two acidic residues have been added, and two basic residues lost, from the positive zone. The negative repeats in bands α_2 and β_2 are also weaker in platelet than skeletal Tm. In all bands the outer non-polar residue repeat seems barely affected. If the repeating pattern of acidic residues is the most important feature of an actin binding site, then not only have one α and one β site been lost through deletion, but it appears that one α and two β sites have been diminished through amino acid substitutions.

In summary, it is clear that platelet Tm has a much lower affinity for actin (whether skeletal or non-muscle) than does skeletal Tm. Under physiological conditions (the free Mg^{++} concentration in skeletal muscle is close to 1 mM) skeletal Tm binds tightly to actin at temperatures less than 30°C; however, at 37°C the affinity of this protein for actin is lowered to the point where binding becomes unstable and very susceptible to small changes in either the temperature or ionic environment (Tanaka, 1972; Wegner, 1979). Presumably, weak binding is essential for the function of skeletal Tm, since it must be able to change its position on the actin filament in response to small changes in conformation transmitted from troponin. Platelet Tm, on the other hand, has been shown to bind poorly to F-actin at physiological ionic strength even when 2.5 mM free Mg^{++} is present

and the temperature is 20°C. (Fig. 42). If the free Mg⁺⁺ concentration in non-muscle cells is also close to 1 mM, then it can be predicted that in vivo non-muscle Tms would bind poorly, if at all, to F-actin filaments. The fact that Tm can be observed attached to actin bundles in non-muscle cells (Lazarides, 1976a,b) indicates that some factor must be capable of enhancing the affinity of platelet Tm for these actin structures.

In Chapter I, the properties of a number of non-muscle proteins capable of forming complexes with F-actin filaments were described. Some of these, either by altering the conformation of the F-actin monomers, or perhaps by interacting directly with the non-muscle Tm, may create a situation in which the binding between Tm and actin becomes stronger. Unfortunately, none of these non-muscle actin binding proteins were available for this study; however, two skeletal muscle proteins with a known ability to interact with actin, myosin S-1 and Tn-I, were both found to enhance the binding of platelet Tm to F-actin.

Myosin S-1, since it does not bind directly to Tm, probably acts to increase the affinity of actin for Tm by altering the conformation of the F-actin monomers (Eaton, 1976). In a complementary sense, it has been shown that the binding of skeletal Tm (or platelet Tm, Chapter VI) to actin increases the affinity of F-actin for myosin heads (Bremel et al., 1973).

Perhaps of more interest is the ability of Tn-I to strengthen the actin-platelet Tm interaction. Many studies, by gel filtration, affinity chromatography, ultracentrifugation, and viscometry, were

unable to demonstrate a direct interaction between Tn-I and skeletal Tm (reviewed by Drabikowski & Dabrowska, 1975). The only direct evidence for an interaction between the two proteins comes from the work of Dabrowska et al. (1976), who found that Tn-I could cause skeletal Tm to form filamentous aggregates and precipitate out of solution.

Actin binding studies provide better, albeit indirect, evidence for an interaction between Tn-I and skeletal Tm. Tn-I is able to bind to F-actin alone, but if skeletal Tm is also present the binding of Tn-I is greater (Potter & Gergely, 1974; Hitchcock, 1975). The reciprocal result, that Tn-I increases the binding of Tm to actin, has been demonstrated for the skeletal (Eaton et al., 1975) and platelet (Fig. 43) proteins.

Further experiments revealed that a complex of Tn-I and Tn-C (Tn-I-C) could bind neither to F-actin nor to skeletal Tm, but would bind to an F-actin-skeletal Tm complex (if Ca^{++} was absent) in an amount not exceeding one mole per seven moles of actin (Hitchcock et al., 1973; Potter & Gergely, 1974; Hitchcock, 1975). Since Tn-C does not bind to F-actin-skeletal Tm it is very likely that the Tn-I-C complex is interacting with the actin-Tm complex through the Tn-I moiety.

The binding studies just described can be interpreted in two ways; either Tn-I binds to a site made up jointly of actin and skeletal Tm, or Tn-I and Tm mutually alter the conformation of F-actin to increase the affinity of each other for the actin filament. The former interpretation is favored, since Tn-I-C binds to F-actin-

skeletal Tm in a one to one molar ratio to skeletal Tm.

The ability of Tn-I to enhance the binding of platelet Tm to actin suggests that platelet Tm retains the binding site for Tn-I. Ohtsuki (1979) has provided evidence to indicate that the site where formerly Tn-T was thought to bind (Fig. 46) is in fact the position at which Tn-I is located. The highly conserved nature of this part of the sequence is certainly consistent with the ability of both skeletal and platelet Tm to interact with Tn-I. It is interesting that this region, which has several unusual features when compared to the rest of the skeletal Tm sequence (Stone et al., 1974; McLachlan & Stewart, 1976b) has remained virtually unchanged in platelet Tm.

As mentioned in Chapter I, no Tn-I like protein has yet been isolated from a non-muscle cell. The ability of both myosin S-1 and Tn-I to increase the binding affinity of platelet Tm to actin suggests, though, that such an ability may be widely shared by other actin binding proteins. Even if there is no Tn-I in non-muscle cells, it is possible that another protein may be able to perform the function of binding platelet Tm to actin filaments. In view of the immunofluorescence studies of Lazarides (1976a,b) it would be particularly interesting to observe the effects of α -actinin on the binding of platelet Tm to actin. The 58k protein from sea urchin eggs, which is capable of organizing actin into highly organized bundles (Bryan & Kane, 1978), may also be a good candidate for a protein able to induce the binding of platelet Tm to actin, since it appears that a portion of this protein extends into the actin groove (Spudich &

Amos, 1979), and thus may be close to the position where x-ray diffraction studies indicate Tm is bound (Huxley, 1973; Haselgrove, 1973).

CHAPTER VI

EFFECTS OF PLATELET TM ON THE ACTIN ACTIVATED ATPase OF MYOSIN S-1

Binding studies, discussed in Chapter V, provided information on the ability of platelet Tm to interact with the other skeletal muscle proteins. The finding that the platelet protein binds weakly to actin, and does not interact at all with Tn-T, produces some doubts as to its ability to function in vivo as a member of a thin filament regulatory system analogous to the one in skeletal muscle.

Further clues as to the role played by platelet Tm in vivo may be obtained by in vitro assays in which the effects exerted by the protein on the actin activated (Mg^{++}) ATPase of myosin are monitored. Such assays have been instrumental in elucidating the biological function of skeletal Tm and each of the three troponin components (Tn-T, Tn-I, and Tn-C). It is generally accepted that the ability of a protein (or any factor) to alter or regulate the actin-myosin interaction in these in vitro assays reflects very closely its ability to exert a similar effect in vivo. It must be assumed that the proteins involved are capable of spontaneously organizing themselves into structures approximating those found in the intact cell.

The ATPase assays described in this chapter were designed chiefly to determine the extent to which platelet Tm could replace skeletal Tm in a skeletal system. In all cases control experiments using skeletal Tm were performed to ensure that the various protein components were active and functioning properly; only then could the

results obtained with platelet T_m be accepted with confidence.

A. SOME PROPERTIES OF THE ACTIN-MYOSIN S-1 SYSTEM

All ATPase assays were performed using skeletal muscle actin and skeletal muscle myosin S-1, a single headed soluble fragment of myosin. The assay buffer, based on that used by Eaton et al. (1975), consisted of 30 mM KCl, 5 mM MgCl₂, 2 mM DTT, 2 mM disodium ATP, 0.1 mM EGTA, and 2 mM Tris. Owing to the binding of Mg⁺⁺ to ATP and ADP this buffer was considered to provide a free Mg⁺⁺ concentration of 3 mM (Mg⁺⁺ not bound to ATP or ADP). For experiments involving a Ca⁺⁺ sensitive response, 0.2 mM CaCl₂ was added to the reaction mixture. Throughout each experiment the pH-stat maintained a pH of 7.8.

A few of the properties of the actin-S-1 system relevant to the experiments that follow will be mentioned here.

The ATPase activity of an actin-S-1 system depends on the concentration of both protein components, so that, for example, the ATPase rate can be kept constant by decreasing the amount of S-1 and increasing the amount of actin. The actual ratio of S-1 to actin does not have any effect on the results obtained with T_m (or T_m-troponin) except at low ATP concentrations, where the rigor binding of S-1 heads to actin filaments can produce an increased rate of ATP hydrolysis. This potentiation of the Mg⁺⁺ ATPase activity of S-1 is more pronounced at high S-1 to actin molar ratios (Bremel et al., 1973). In certain cases where the potentiation made it difficult to measure accurate ATPase rates, it was reduced either by raising the initial ATP concentration to 4 mM, or by increasing the actin to S-1 ratio.

The actin to S-1 molar ratios in the experiments in this chapter varied from 1:1 to 3:1, with protein concentrations kept such that the ATPase rates were in the range of 0.25 to 0.50 μ moles PO_4^{2-} /minute.

In the absence of Tm , the actin activated S-1 ATPase activity was found to be constant with respect to time, levelling off only when the ATP was exhausted. That this rate is independent of ATP concentration over a wide range (10^{-5} to 10^{-3} M) is well documented (Bremel et al., 1973; Eaton et al., 1975). A slight increase in activity as the reaction proceeded was sometimes noticed, probably a result of the small decrease in salt concentration (approximately 3 mM by the end of a typical assay) produced by the continual addition of low ionic strength KOH (10 to 15 mM) as a titrant to compensate for the release of protons during ATP hydrolysis.

The ATPase activity of an actin-S-1 system is highly dependent on the salt concentration, rising rapidly as the ionic strength drops. It is not, however, greatly affected by dilution with buffer of the same ionic strength. The addition of 0.5 ml of assay buffer to an ATPase assay containing actin and S-1 in a 2 ml volume had no effect on the rate of ATP hydrolysis. This shows that the addition of a protein solution during the course of an assay does not, by simple dilution, alter the ATPase rate of S-1. To be certain that changes in the ATPase activity of S-1 were not a result of variations in the ionic strength, all proteins to be used in the assays were dialyzed overnight against the assay buffer (minus ATP).

Further control experiments demonstrated that Ca^{++} , in the amounts needed for experiments involving Ca^{++} sensitivity (0.2 mM),

did not significantly affect the S-1 ATPase rate. The addition of Ca^{++} to the assay always led to the immediate displacement of protons from EGTA, resulting in a drop in pH, which could appear as an increase in the rate of ATP hydrolysis.

S-1 by itself, without actin, had a small but measurable ATPase activity under the assay conditions. No corrections were made for this activity (about 0.035 $\mu\text{moles PO}_4/\text{minute/mg S-1}$) in the experiments described in this chapter. Such a correction would slightly improve the percent inhibitions recorded here.

Variations in activity of $\pm 10\%$ could occur between two assays to which had been added the same amounts of S-1 and actin. Therefore, for accurate results, the initial actin activated S-1 ATPase rate was always measured for each assay prior to the addition of other proteins. The percent inhibition or activation produced by the subsequent addition of proteins could then be calculated directly using this rate. For examples of this method see Figs. 48 and 57. Repeated experiments confirmed the fact that there was no need to preincubate Tm or any of the troponin components with actin; the full activity of all proteins was expressed almost immediately upon addition to the S-1-actin system.

B. PLATELET TM ALONE

Skeletal Tm, by itself, if it is bound to actin, is able to exert an effect on the actin activated S-1 ATPase (Eaton et al., 1975). At high concentrations of ATP, where the myosin heads are saturated, skeletal Tm can inhibit the S-1 ATPase by up to 60% (Figs. 48 and 49). Platelet Tm, under the conditions used for the experiment in Fig. 49

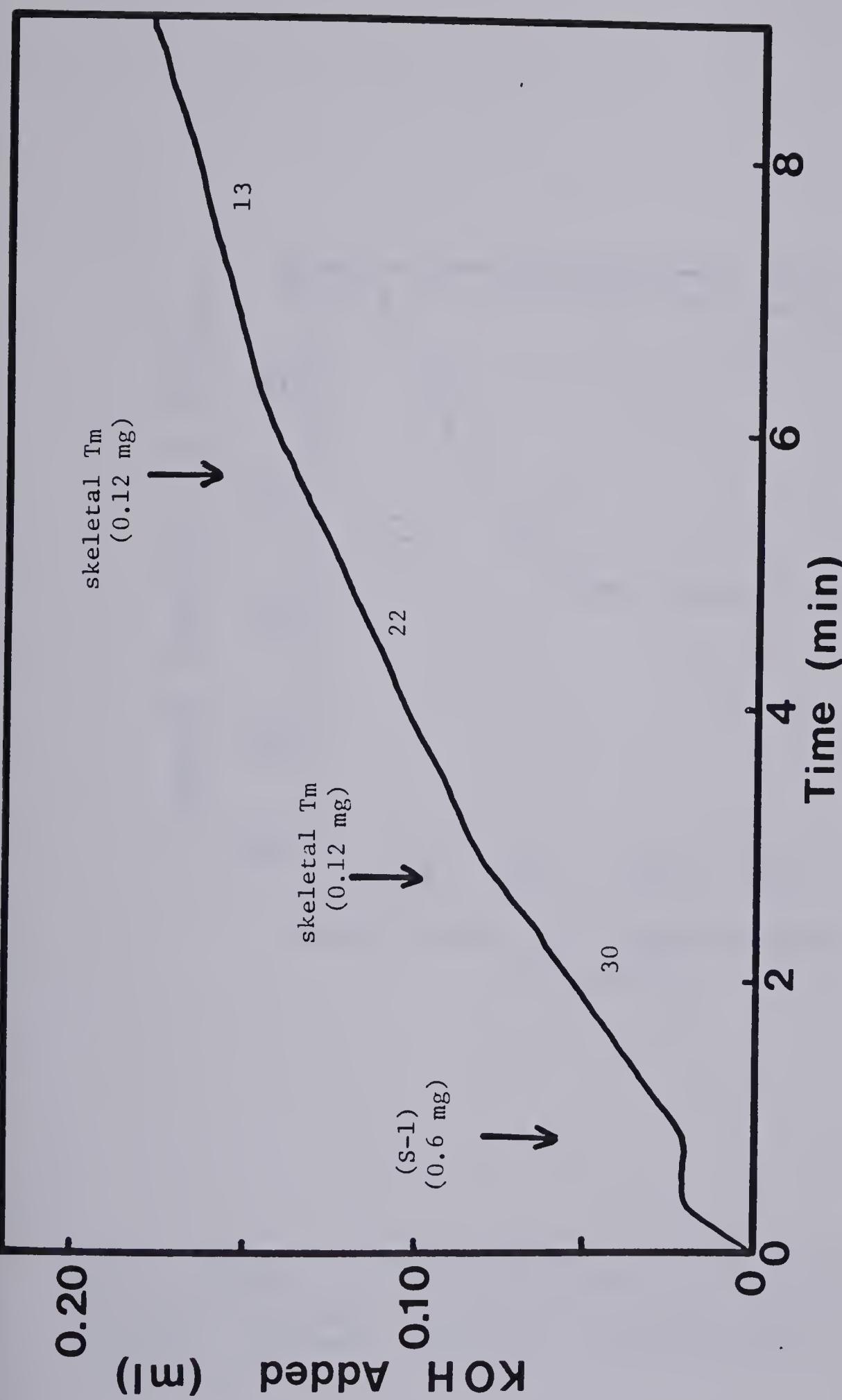


Fig. 48. Example of a plot, as produced by the pH-stat, showing the volume of KOH added to an actin-S-1 ATPase assay as a function of time. The experiment was initiated by adding S-1 to an F-actin (0.66 mg) solution containing ATP which had been adjusted to pH 7.8 by the pH-stat. When a linear velocity vs. time plot, allowing an accurate measurement of the activity, had been produced, an aliquot of skeletal Tm was added. Inhibition of the S-1 ATPase occurred almost immediately, resulting in a straight line with decreased slope. The numbers beneath the line give the activity in μl KOH added per minute. The amount of inhibition is calculated from the slope of the line before and after addition of Tm. A second aliquot of Tm produced further inhibition, which again could be quantitated by comparison with the original actin-S-1 slope. The assay buffer contained 30 mM KCl, 5 mM MgCl₂, 2 mM DTT, 2 mM disodium ATP, 2 mM Tris, 0.1 mM EGTA. Results of this assay and others are collected in Fig. 49.

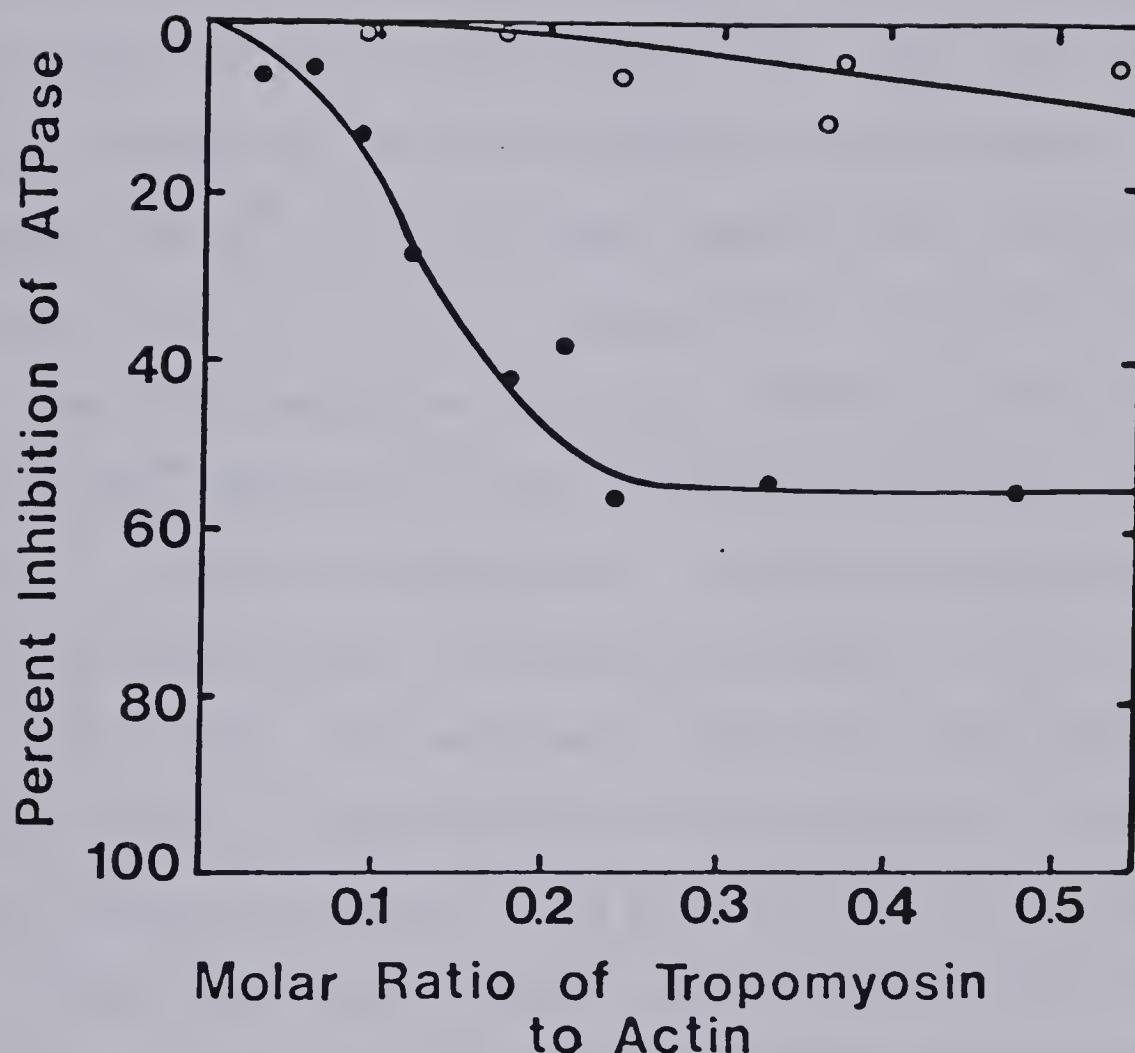


Fig. 49. Effect of platelet (O) and skeletal α (●) Tm on the actin activated ATPase of S-1 in the presence of 3 mM free Mg^{++} . The assay contained 0.66 mg actin and 0.6 mg S-1 in 2 ml of the standard buffer (30 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 2 mM disodium ATP, 0.1 mM EGTA, and 2 mM Tris, pH 7.8).

(3 mM free Mg⁺⁺), binds only poorly to actin, and as a consequence, had almost no effect on the S-1 Mg⁺⁺ ATPase.

Increasing the free Mg⁺⁺ concentration to 8 mM produces conditions under which platelet Tm can bind to actin (Fig. 40). The protein then exhibited an ability to inhibit the actin activated S-1 ATPase by 40% at a 1 or 2:7 molar ratio to actin (Fig. 50). Skeletal Tm once again displayed the maximal inhibitory effect (60%) it had achieved at 3 mM Mg⁺⁺, but did produce slightly better inhibition at lower molar ratios to actin, presumably due to tighter binding.

When ATP is present at low levels, skeletal Tm can actually enhance the Mg⁺⁺ ATPase rate of S-1 (Shigekawa & Tonomura, 1972; Eaton et al., 1975); the extent of the activation being dependent on the ratio of myosin heads to actin monomers (Spudich & Watt, 1971; Bremel et al., 1973). The low levels of ATP allow many myosin heads to bind to F-actin in rigor complexes (nucleotide free S-1 has a very high affinity for actin, with a K_D⁻⁷ M or lower (Bremel et al., 1973)), and it is thought that the presence of these rigor bonds, in conjunction with Tm, somehow alters the conformation of the F-actin monomers so that their cofactor activity (ability to activate the remaining free S-1) is potentiated. The effect can be observed as an upward curve on the velocity versus time plot as the amount of ATP remaining approaches zero (Fig. 51).

With skeletal Tm, the rate of ATP hydrolysis, determined from the slope of the plot in Fig. 51, increased from an inhibited value at 1 to 2 mM ATP of 0.17 (μ moles PO₄/minute/mg S-1) to a maximum value of 0.61 at an ATP concentration of 0.1 mM. Platelet Tm

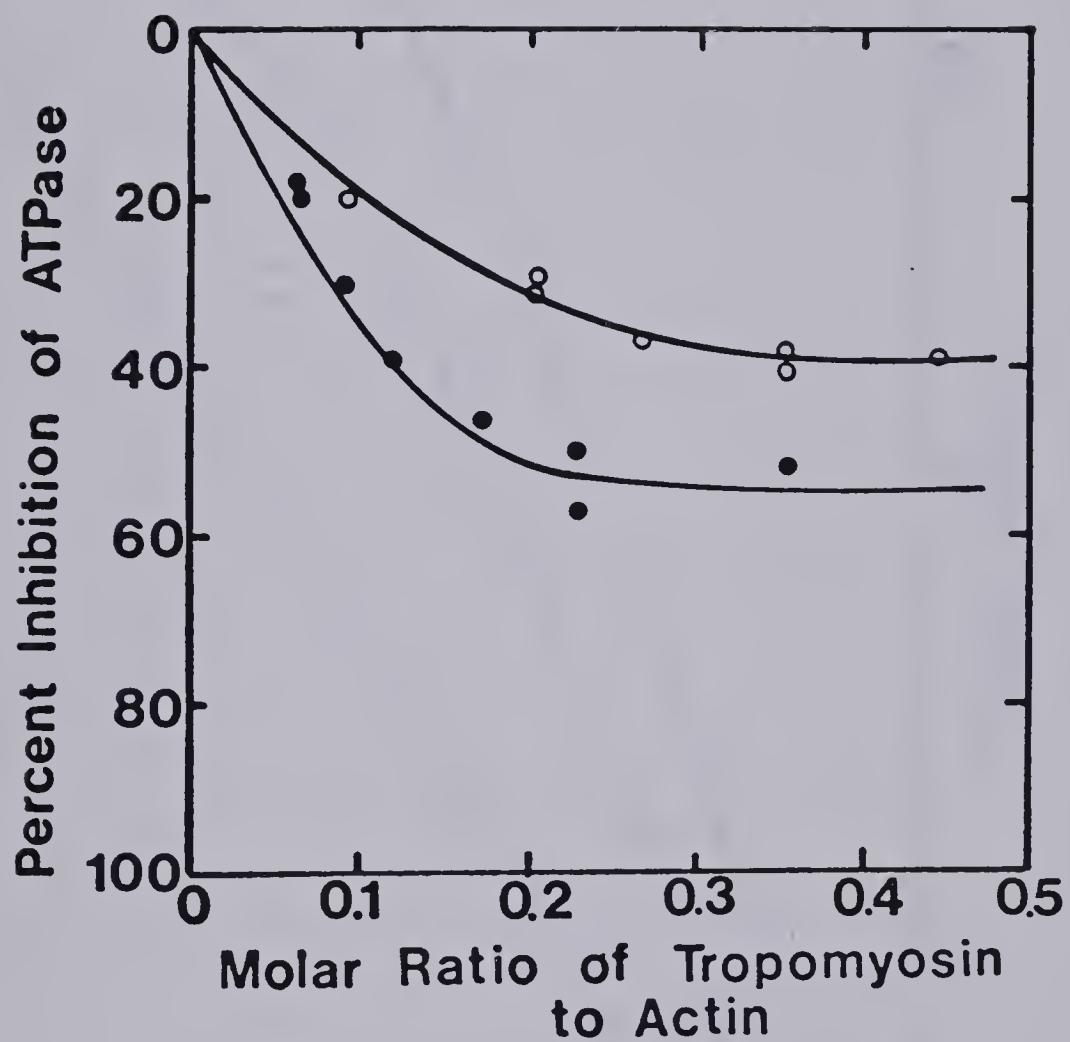


Fig. 50. Effect of platelet (○) and skeletal α (●) Tm on the actin activated ATPase of S-1 in the presence of 8 mM free Mg⁺⁺. The assay contained 0.56 mg actin and 1.08 mg S-1 in 2 ml of the standard buffer, except that the MgCl₂ concentration was raised to 10 mM.

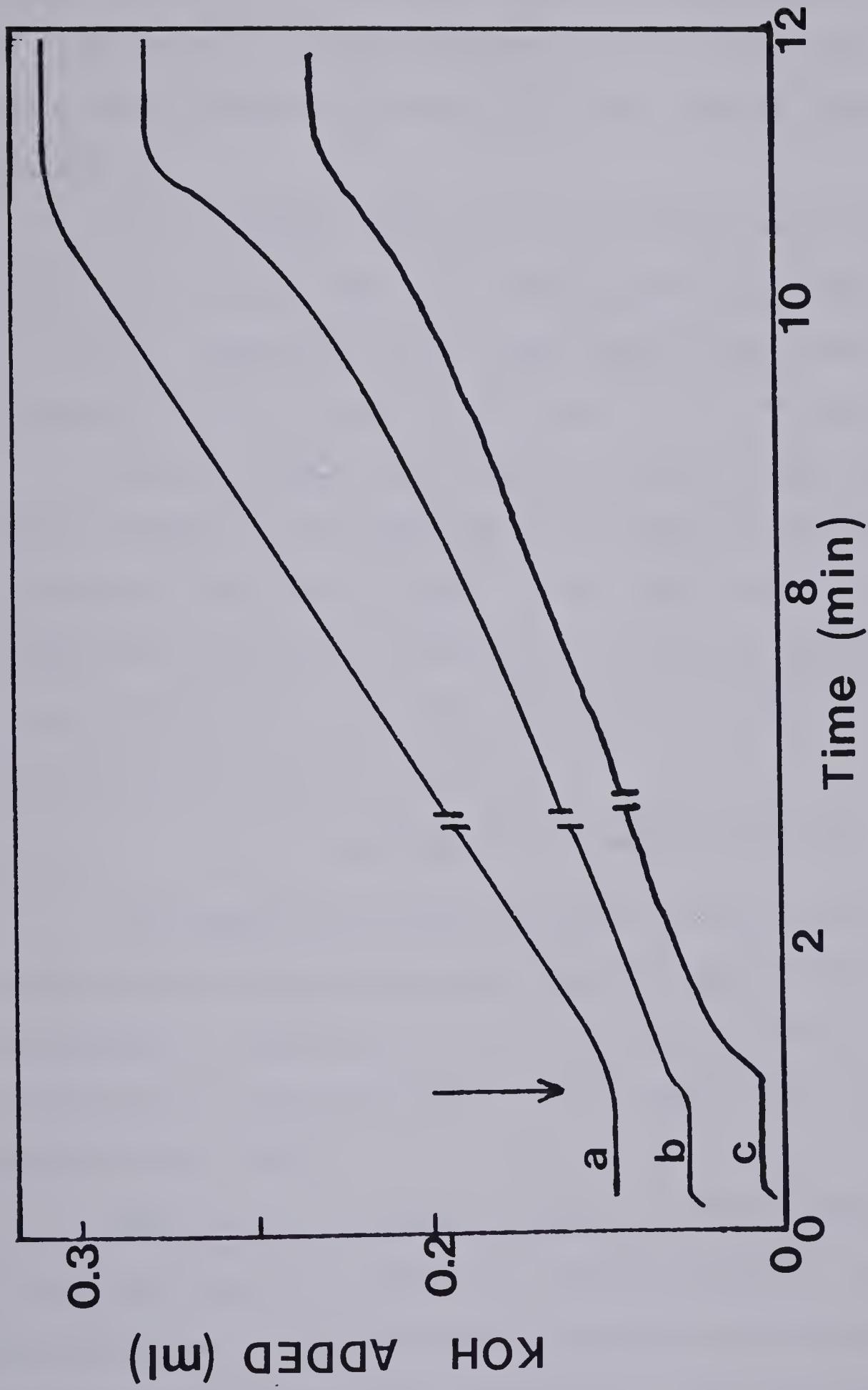


Fig. 51. ATPase assays with S-1 and actin alone (a), with skeletal Tm (b), and with platelet Tm (c), showing the effects on the ATPase activity of S-1 as the concentration of ATP decreases with time. With the concentration of KOH known, the amount of ATP remaining at any time can be determined by extrapolating back from the point where the reaction ceases (0 mM ATP). The assay contained 1.0 mg S-1, 0.25 mg actin, and where added 0.11 mg of either platelet or skeletal Tm. The standard buffer was used except in the assay with platelet Tm where 9 mM MgCl₂ was present.

increased the initial rate of the Mg^{++} ATPase of S-1 from 0.14 to a maximum of 0.32 at 0.1 mM ATP. This latter experiment was performed in 7 mM free Mg^{++} , so that platelet Tm would be fully bound to actin, and capable of inhibiting the S-1 Mg^{++} ATPase rate at high ATP concentrations.

Under conditions where platelet Tm did not bind well to actin, a small upturn (about 50%) in S-1 ATPase activity was still observed at 0.1 mM ATP. Presumably, at this point, enough myosin heads were attached to actin to induce the binding of platelet Tm (see Fig. 44).

Thus platelet Tm, like skeletal Tm, exerts a dual effect on the Mg^{++} ATPase of S-1: inhibition at high levels of ATP followed by activation at low levels of ATP. In both cases, however, changes brought about by platelet Tm appear to be smaller in magnitude than those of skeletal Tm, even under conditions where saturation of actin with platelet Tm should have occurred.

C. PLATELET TM WITH SKELETAL TROPONIN

The inhibition imposed by skeletal Tm on the actin activated ATPase of S-1 is increased upon addition of troponin, and, more importantly, is rendered Ca^{++} sensitive; that is, release of the inhibition occurs in the presence of Ca^{++} (Ebashi et al., 1968; Spudich & Watt, 1971).

For troponin to function effectively it is essential that it contain all three of its subunits in equimolar amounts. Troponin preparations with unequal amounts of the three components are often unable to confer complete Ca^{++} sensitivity. Many preparations tested in this study were quite insoluble at low ionic strengths, and,

although they inhibited well in the absence of Ca^{++} , they did not fully release this inhibition in the presence of Ca^{++} . In some cases, this type of behaviour may have been due to a troponin poor in Tn-C (Drabikowski et al., 1973), and may have been caused by dialyzing the troponin, after the final ammonium sulfate precipitation, against buffer containing EGTA. The absence of Ca^{++} greatly weakens the binding between Tn-C and the other components, perhaps allowing them to dissociate (Perry et al., 1973; Ebashi et al., 1973). The troponin preparations used in this study (Fig. 52) were dialyzed, after purification, against H_2O with only mercaptoethanol present, and were able to confer very good Ca^{++} sensitivity to the actin-S-1 system in the presence of skeletal Tm.

In the absence of Ca^{++} skeletal Tm and troponin inhibited the Mg^{++} ATPase of S-1 by up to 80%, but when Ca^{++} was added this inhibition was fully released, and in certain cases significant activations were observed (Fig. 53 and 54).

Platelet Tm was capable of interacting with troponin to inhibit the actin activated S-1 ATPase, but was only partially able to release this inhibition in the presence of Ca^{++} (Figs. 53, 54, and 55). This result was obtained with three different preparations of rabbit skeletal muscle troponin, all of which yielded full Ca^{++} sensitivity in the presence of skeletal Tm, and with platelet Tm prepared by both methods 1 and 2. The same effect was observed at a 1:7 (Fig. 53) and 3:7 (Fig. 54) molar ratio of troponin to actin at all platelet Tm concentrations, and at a constant platelet Tm concentration regardless of the troponin concentration (Fig. 55).

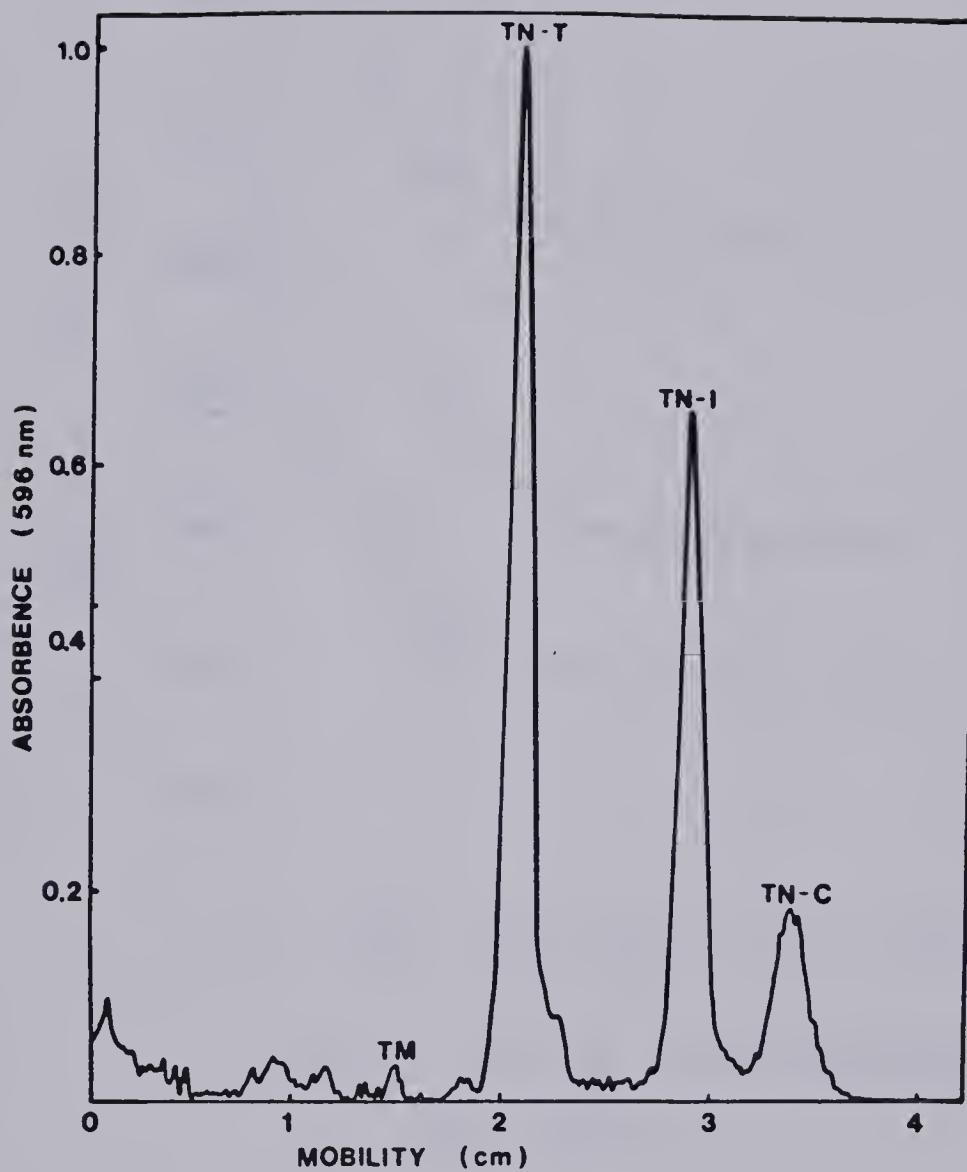


Fig. 52. An SDS gel, scanned at 596 nm, of the troponin preparation used in experiments shown in Fig. 53 & 54. There is very little contamination with skeletal Tm.

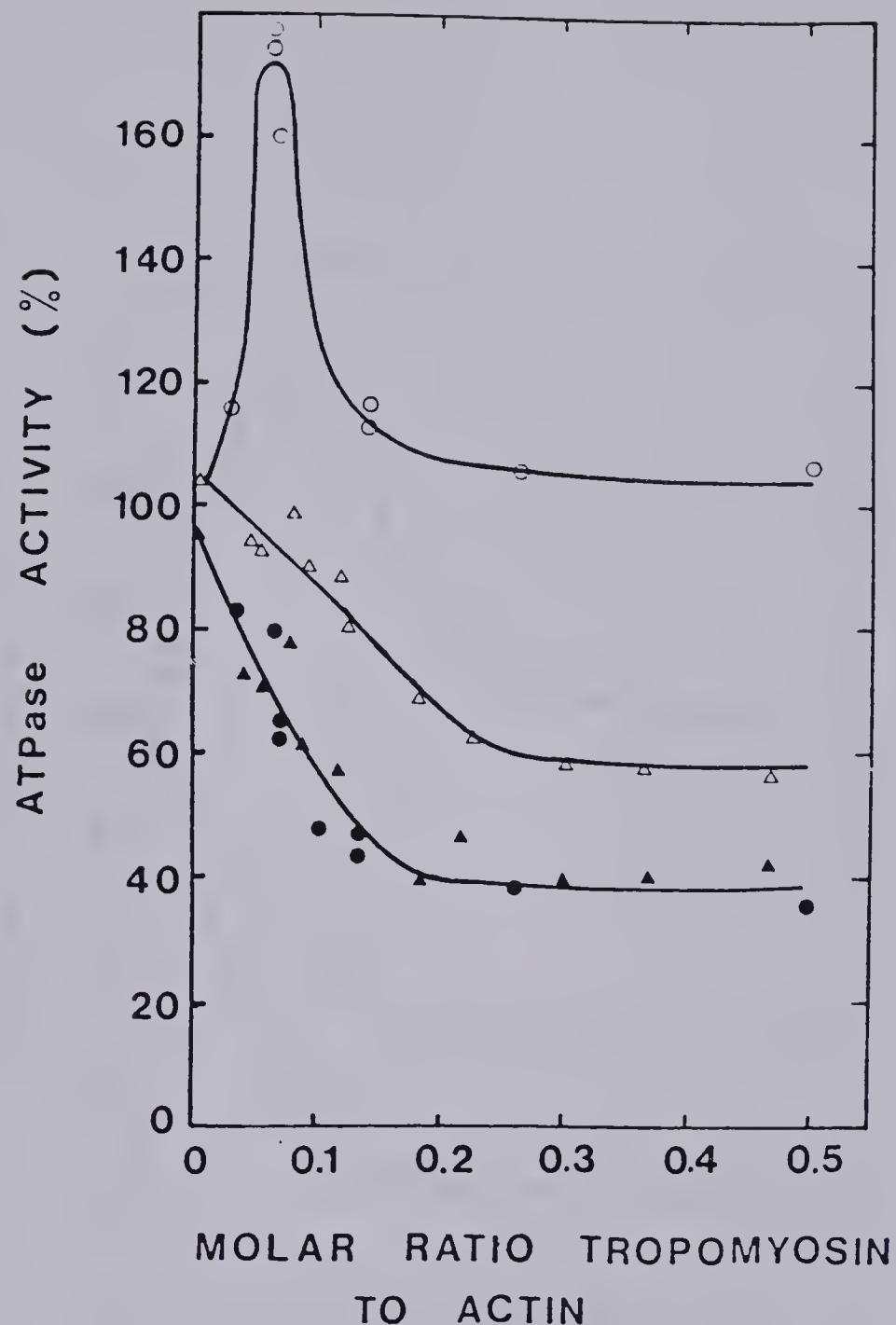


Fig. 53. Effect of increasing amounts of cardiac (○,●) and platelet Tm (Δ , \blacktriangle) on the actin activated S-1 ATPase activity in the presence of a 1:7 molar ratio of troponin to actin, either with (○, Δ) or without Ca^{++} (●, \blacktriangle). Assays contained 0.27 mg/ml of actin and 0.4 mg/ml of S-1 in the standard buffer.

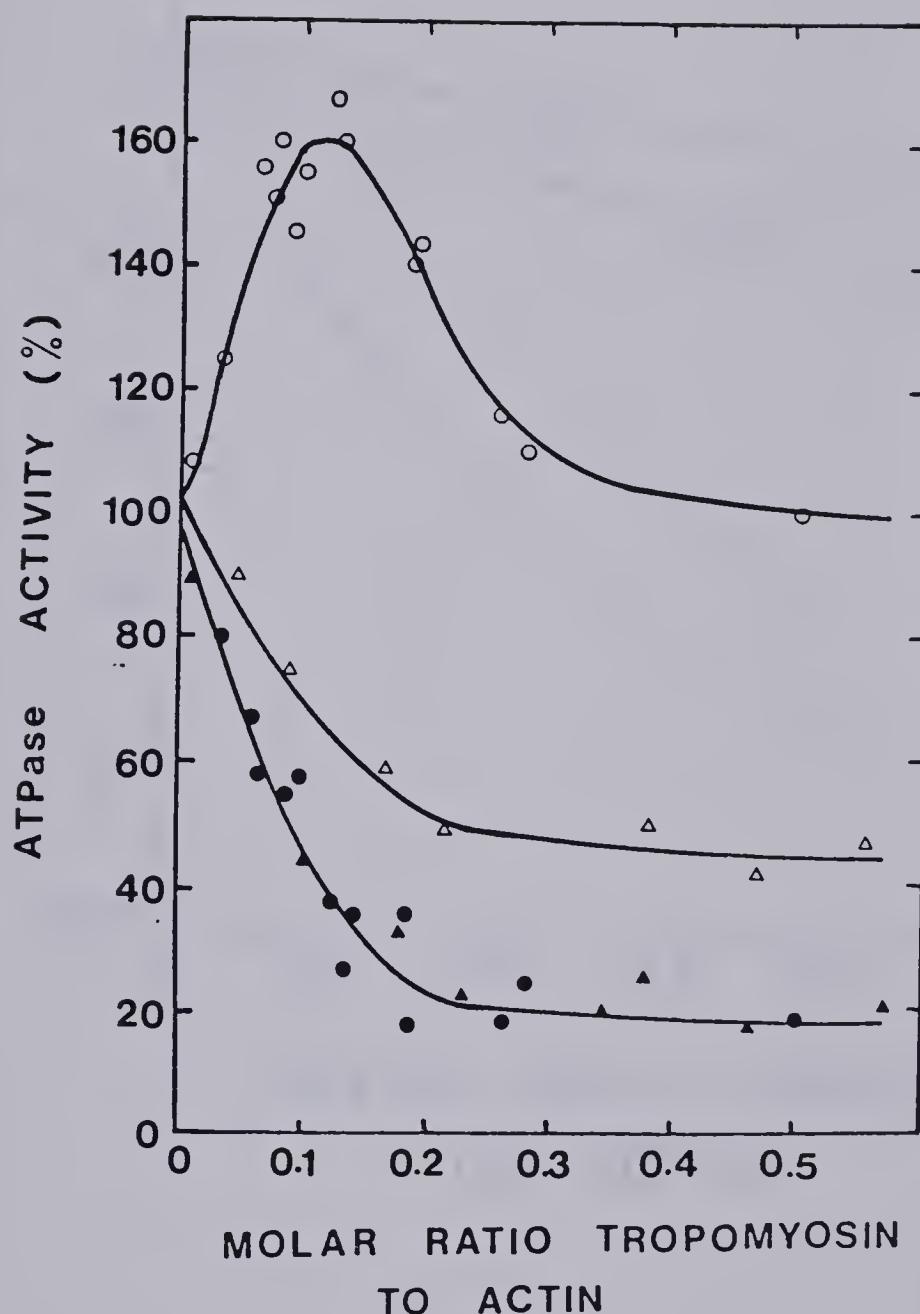


Fig. 54. Effect of increasing amounts of cardiac (O,●) and platelet (Δ , \blacktriangle) Tm on the actin activated ATPase of S-1 in the presence of a 3:7 molar ratio of troponin to actin, either with (O, Δ) or without (●, \blacktriangle) Ca^{++} . Assays contained 0.35 mg/ml actin and 0.4 mg/ml S-1 in 2 ml of the standard buffer.

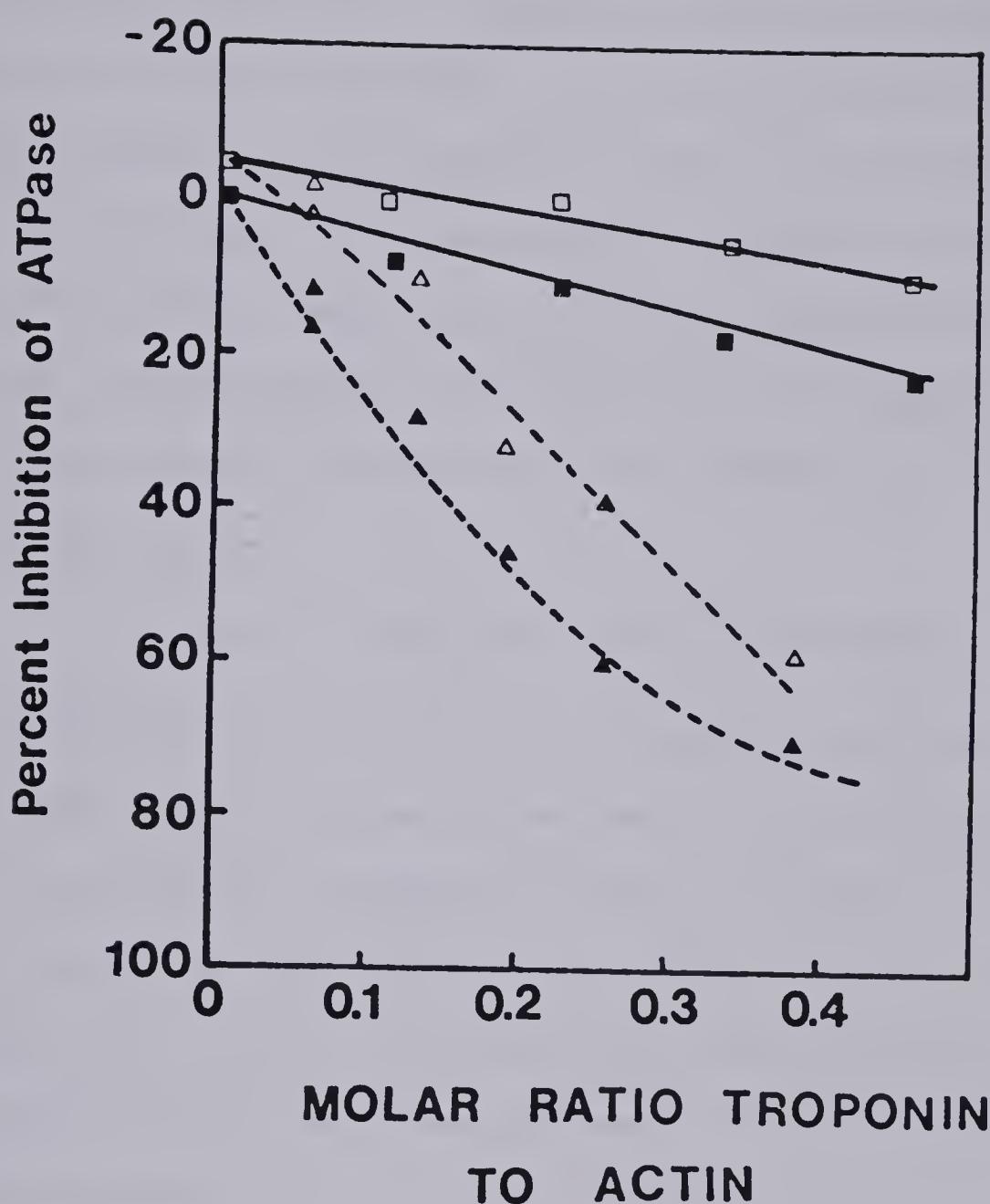


Fig. 55. Effect of increasing amounts of troponin on the actin activated ATPase of S-1 in the absence (\square , \blacksquare) and presence (\triangle , \blacktriangle) of platelet Tm. The assay contained 0.29 mg/ml actin, 0.4 mg/ml S-1, and where added 0.09 mg/ml platelet Tm in 2 ml of the standard buffer. Points marked (\blacksquare , \blacktriangle) were in the absence, while those marked (\square , \triangle) were in the presence of Ca^{++} .

These assays were all performed under conditions (3 mM free Mg^{++}) where platelet Tm does not bind well to actin by itself, reliance being placed upon the ability of troponin to induce this binding (probably via the Tn-I component). To ensure that the poor Ca^{++} sensitivity which was observed was not a result of the weak binding of platelet Tm to actin, the experiment was repeated at a free Mg^{++} concentration of 8 mM. Again, full Ca^{++} sensitivity was observed with cardiac Tm and troponin, but platelet Tm in the presence of troponin inhibited and did not release this inhibition upon addition of Ca^{++} (Table XVIII).

D. PLATELET TM WITH THE SUBUNITS OF TROPONIN

A number of studies have been performed on the effects which the individual troponin components have on the actin activated ATPase of S-1 in the presence of skeletal Tm (Greaser & Gergely, 1971; Shigekawa & Tonomura, 1973; Ebashi et al., 1973; Eisenberg & Kielly, 1974; Eaton et al., 1975). In general, the results obtained by these different groups are in good agreement with each other, and can be summarized as follows.

The Tn-C or Tn-T subunits, individually, have little or no effect on the S-1 ATPase, while Tn-I, or a combination of Tn-I and Tn-T, inhibit regardless of the Ca^{++} concentration. A mixture of Tn-C plus Tn-T, depending on the study cited, can display either a small degree of positive or negative Ca^{++} sensitivity or no effect at all. The variability of the results with Tn-C and Tn-T is possibly a consequence of the highly insoluble nature of the Tn-T component of this complex in the absence of Ca^{++} .

TABLE XVIII

Effects of Cardiac and Platelet Tm on the Actin-Activated
ATPase of S-1 in the Presence of Troponin and
8 mM Free Mg⁺⁺

Components Added ^a	<u>Percent ATPase Activity</u>	
	Without Ca ⁺⁺	With Ca ⁺⁺
None	100	
Cardiac Tm + Troponin	20	96
Platelet Tm + Troponin	22	39
Cardiac Tm	66	
Platelet Tm	73	

^a The assay buffer was 30 mM KCl, 10 mM MgCl₂, 2 mM DTT, 2 mM ATP, 0.1 mM EGTA, 2 mM Tris; for + Ca⁺⁺ 0.2 mM CaCl₂ was added. The assay contained 1.0 mg S-1, 0.64 mg actin and where added 0.22 mg platelet Tm, 0.28 mg troponin and 0.25 mg cardiac Tm.

1. Platelet Tm with Tn-I

The addition of Tn-C to an assay containing platelet Tm, actin, and S-1 had no effect on the ATPase activity of S-1. Tn-I though was able to interact with platelet Tm to inhibit the Mg^{++} ATPase of S-1 (Figs. 56a,b). Such inhibition could only occur if platelet Tm was bound to the F-actin filaments, confirming the results of the binding assays (Chapter V), in which the ability of Tn-I to induce the interaction of platelet Tm with actin was demonstrated.

At a 1:7 Tn-I to actin molar ratio platelet Tm does not inhibit nearly as well as skeletal Tm, but at a 3:7 molar ratio the two Tms display very similar inhibitory properties (because of its larger size skeletal Tm should always completely inhibit at a lower molar ratio (by 6/7) than platelet Tm).

The ability of Tn-I to induce the binding of skeletal Tm to actin has been shown to decrease as the solution conditions become less favourable for the actin-Tm interaction (Eaton et al., 1975).

It is expected that if the Mg^{++} concentration were raised so that the platelet Tm-actin interaction was strengthened, a lower molar ratio of Tn-I to actin would be sufficient to produce complete inhibition with platelet Tm. It is likely that in Fig. 56b there is only one Tn-I bound per platelet Tm (six actins), and the excess Tn-I is only necessary in order to push the equilibrium in favour of the bound actin-Tn-I-platelet Tm complex.

Experiments involving the addition of Tn-T to an actin-S-1 system incorporating platelet Tm were not performed, but it can be assumed that little if any alteration in the ATPase activity would

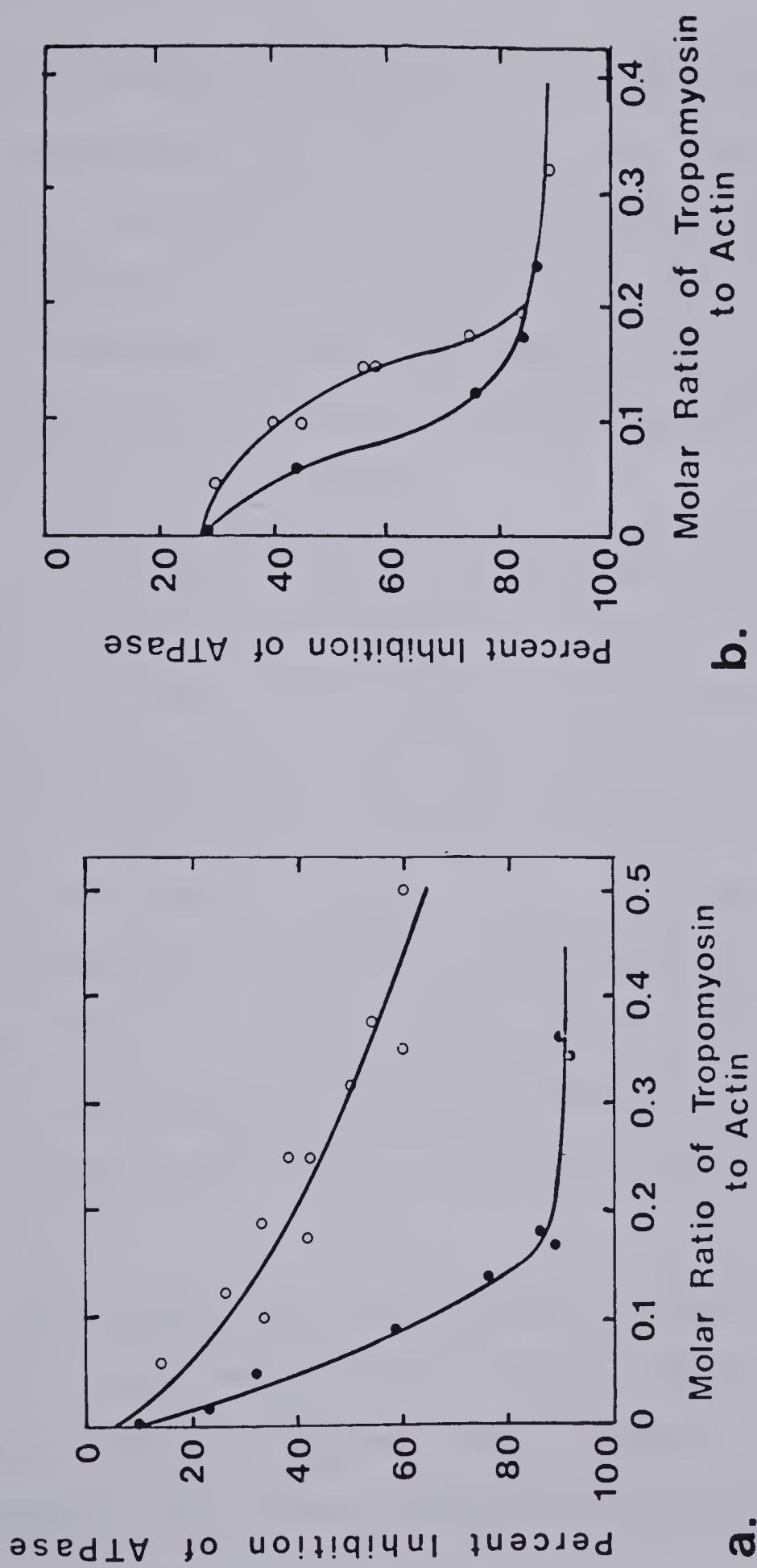


Fig. 56a,b. Effects of platelet (○) and skeletal α (●) Tm on the actin activated ATPase of S-1 in the presence of (a) a 1:7 molar ratio and (b) a 3:7 molar ratio of Tn-I to actin. The assays contained 0.72 mg actin and 0.67 mg S-1 in 2 ml of the standard buffer.

result, as Tn-T binds neither to platelet Tm (Chapter V), nor to actin (Potter & Gergely, 1974).

2. Platelet Tm and a Complex of Tn-I with Either Tn-C or Calmodulin

The last combination of troponin subunits to be discussed is that of Tn-I with Tn-C (Tn-I-C). This is perhaps the most interesting case with regards to platelet Tm, since platelet Tm does not bind to Tn-T, and furthermore, certain experiments in the literature imply that a Ca^{++} sensitive inhibition of the actin activated S-1 ATPase is possible in the absence of Tn-T.

Tn-I and Tn-C can bind to each other in the absence of Ca^{++} but the strength of the interaction is increased if Ca^{++} is present (Perry et al., 1973). The varying affinities of the two proteins for each other probably explains why Tn-I-C binds in a Ca^{++} sensitive manner to an actin-skeletal Tm complex (Hitchcock et al., 1973; Hitchcock, 1975; Potter & Gergely, 1974). The experiments by these groups, performed under conditions where skeletal Tm by itself binds to actin (0.05 to 0.10M KCl, 2 mM free Mg^{++}), showed that one mole of Tn-I-C per Tm molecule was associated with the actin-Tm complex in the absence of Ca^{++} , while no interaction occurred in the presence of Ca^{++} .

These results raise the possibility that some degree of Ca^{++} sensitivity may be conferred to an actin-S-1 system by a combination of these two troponin components, working together with skeletal Tm, without need of Tn-T. These expectations are confirmed in part by the results of ATPase assays.

It was found that inhibition in the presence of Tn-I-C

(minus Ca^{++} , plus skeletal Tm) did occur, although the amounts of Tn-I-C required to decrease the actin activated S-1 ATPase by a significant amount (60 to 70%) were quite high, ranging from a 0.5:1 (Eaton et al., 1975) to a 1:1 (Eisenberg & Kielly, 1974) to a 2:1 (Perry et al., 1973; Ebashi et al., 1973) molar ratio to actin. The addition of Ca^{++} always had the same effect of completely abolishing inhibition, which meant that it was in fact possible to observe a Ca^{++} sensitive regulation of the actin-myosin interaction without Tn-T, providing that enough Tn-I-C was present.

On closer examination, however, it becomes apparent that the various binding and ATPase studies described in the literature cannot be directly compared, since the binding assays were performed under conditions where skeletal Tm binds to actin, while all the ATPase assays were performed under conditions (0 mM free Mg^{++}) where skeletal Tm does not bind to actin (Eaton et al., 1975). The ATPase assays therefore do not reflect the true situation in skeletal muscle, where Tm should, by itself, be able to associate with actin.

Experiments were undertaken to determine the effects of Tn-I-C on an ATPase assay under conditions where skeletal Tm was capable of binding to actin (3 mM free Mg^{++}). An example of how these experiments were performed is given in Fig. 57. Results of a number of assays, performed at varying concentrations of Tn-C, revealed that in the presence of Ca^{++} the Tn-I-C complex plus skeletal Tm rate did not rise above the inhibited rate observed with Tm alone (Fig. 58).

Unlike the ATPase assays in the literature, these results agree well with the binding studies of Hitchcock et al. (1973) and Potter

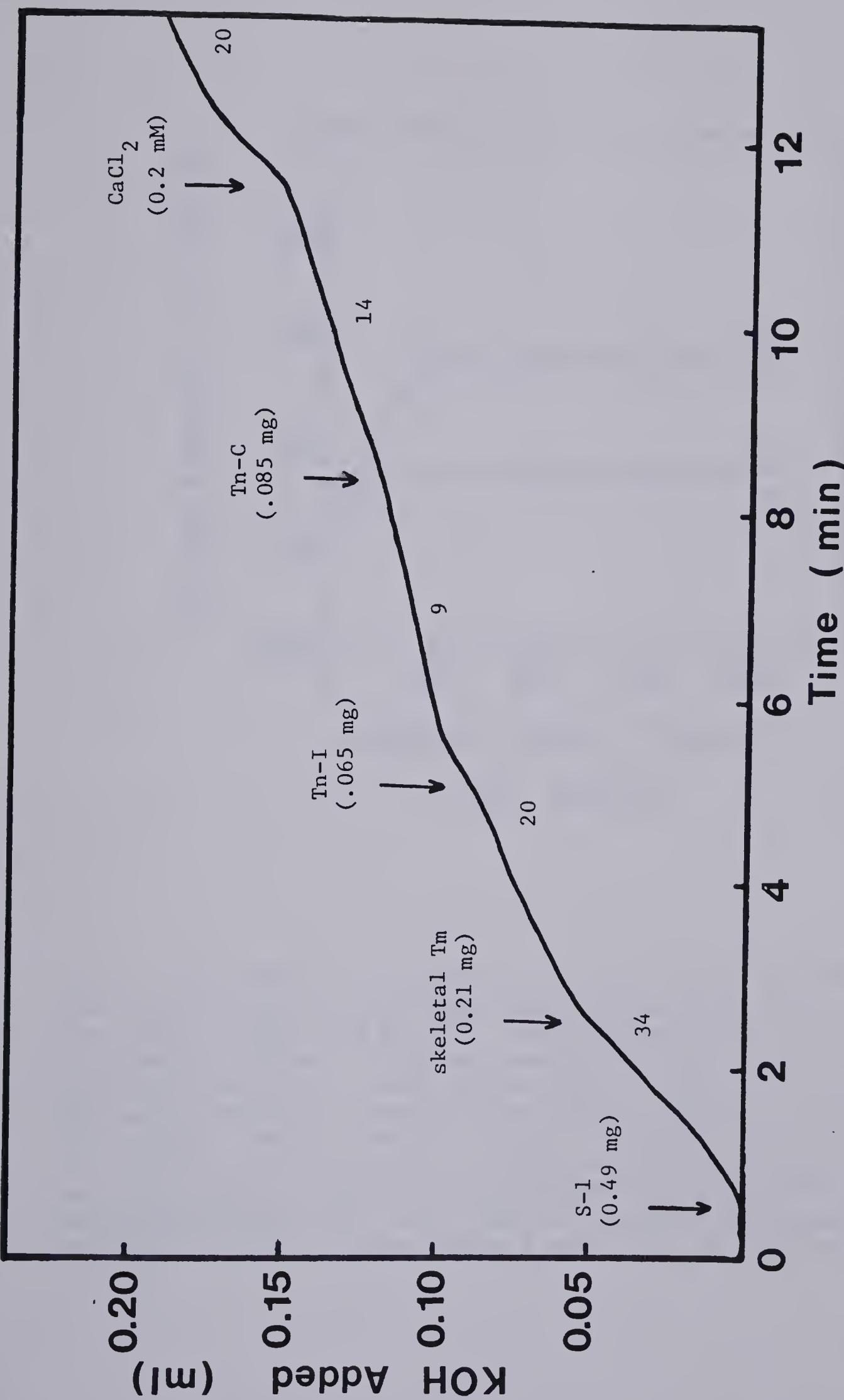


Fig. 57. Example of a plot, as produced by the pH-stat, showing the volume of KOH added to an actin-S-1 ATPase system as a function of time. The position at which proteins were added and their amounts are indicated on the graph. The number beneath the line gives the slope in $\mu\text{l KOH per minute}$ at that point. The assay contained 0.5 mg actin in 2 ml of the standard buffer, and was initiated upon addition of S-1. By performing assays in this manner the $\text{Tn-I-C plus Ca}^{++}$ rate can be compared directly to the plus Tm rate previously measured for the same assay.

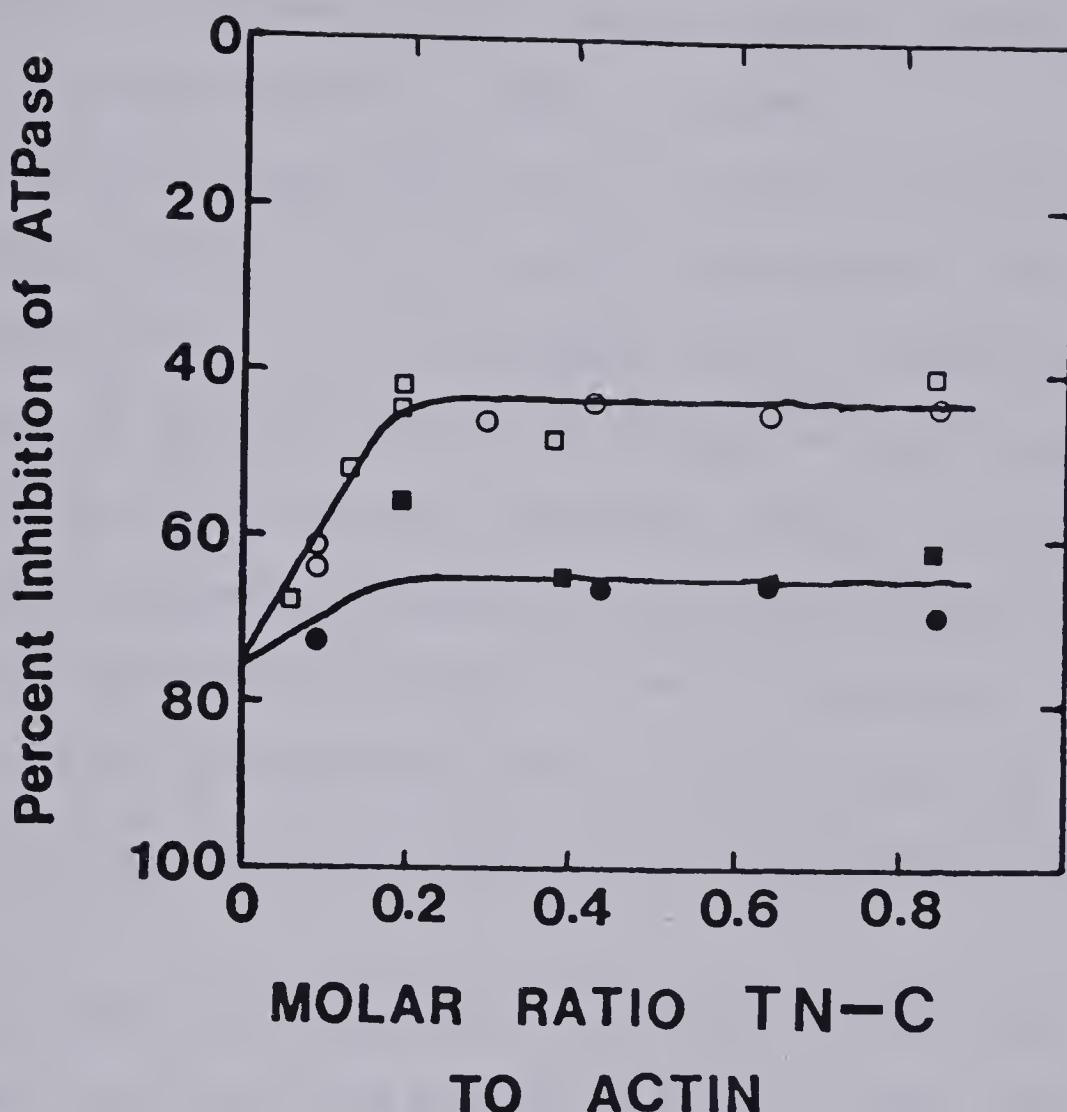


Fig. 58. The effect of increasing amounts of Tn-C on the actin activated ATPase of S-1 in the presence of skeletal Tm and Tn-I, either with (\square , \circ) or without (\blacksquare , \bullet) Ca^{++} . Assays marked (\circ , \bullet) contained 0.5 mg actin, 0.49 mg S-1 and were performed in the standard buffer containing 4 mM MgCl_2 . Assays marked (\square , \blacksquare) contained 0.25 mg actin, 0.98 mg S-1 and were performed in the standard buffer with 6 mM MgCl_2 and 4 mM ATP. Skeletal Tm was held at a 0.19 molar ratio and Tn-I at a 0.24 molar ratio to actin. Inhibition with skeletal Tm alone was near 40%, while the inhibition with skeletal Tm and Tn-I was near 75%. The inhibition with Tn-I, Tn-C, plus Ca^{++} , plus Tm was identical to the inhibition with Tm alone, above a molar ratio of Tn-C to Tn-I of 1:1.

and Gergely (1974). It is, of course, obvious from the binding studies that the state of an actin filament (and therefore the ATPase activity of S-1) should be the same in the presence of Tn-I-C, skeletal Tm, and Ca^{++} , as in the presence of skeletal Tm alone.

In order to obtain these results care had to be taken to avoid the activation (potentiation) of the Mg^{++} ATPase of S-1, which occurs at low ATP concentrations in the presence of Tm. If such activation had occurred, it would have appeared as though the Tn-I-C complex, plus Ca^{++} , plus skeletal Tm rate (measured at the end of the assay, with little ATP remaining) was greater than the S-1 ATPase rate observed with Tm alone (measured at the start of the assay). To lessen the effects of potentiation assays were performed either at high actin to S-1 molar ratios (3:1), or at high initial ATP concentrations (4 mM).

To summarize then, full Ca^{++} sensitivity can not occur with Tn-I-C under conditions where skeletal Tm binds to actin. The partial (30%) Ca^{++} sensitivity found is the result of a difference in inhibitory power between the Tn-I-C plus skeletal Tm complex (- Ca^{++}), and skeletal Tm alone, which is all that remains bound to actin after Ca^{++} is added.

It was mentioned that some Ca^{++} sensitivity could be obtained with Tn-I-C and skeletal Tm, if conditions were such that skeletal Tm alone could not bind to actin. This may be explained as follows.

Tn-I-C is able to hold skeletal Tm onto the actin filament in the absence of Ca^{++} (Eaton et al., 1975), however, the binding of Tn-C to Tn-I greatly weakens the ability of Tn-I to produce inhibition, so that only a small amount of inhibition results, even though all

three proteins are bound to actin. The addition of Ca^{++} will release the Tn-I-C complex from the thin filament (Hitchcock et al., 1973; Potter & Gergely, 1974), skeletal Tm will then also detach, and release of this inhibition will occur.

At low molar molar ratios of Tn-I to actin, it is clear that only poor Ca^{++} sensitivity is obtained with Tn-I, Tn-C and skeletal Tm in the absence of Mg^{++} . From Fig. 59, it would also appear that the Tn-I-C complex is not nearly as effective as Tn-I alone is at inducing the binding of skeletal Tm to actin. Perhaps this is why such high ratios of Tn-I-C are needed before good inhibition is observed.

Very similar results are obtained with platelet Tm in 3 mM free Mg^{++} (Fig. 59). Tn-I-C, either with or without Ca^{++} , is not an effective inducer of the binding of platelet Tm to actin. Perhaps if much higher ratios of Tn-I-C had been used (1:1 or 2:1 molar ratio to actin) binding may have been observed (- Ca^{++}). The lack of binding is reflected in the results of the ATPase assays (Fig. 60). Very little inhibition is observed even in the absence of Ca^{++} .

Platelets and non-muscle cells in general do not contain Tn-C, but do contain large amounts of another Ca^{++} binding protein, now usually called calmodulin (Watterson et al., 1976; Stevens et al., 1976; Muszbek et al., 1977; Grand et al., 1979). Calmodulin interacts with Tn-I in the presence of Ca^{++} , although the complex formed (Tn-I-Cal) is less stable than the Tn-I-C complex (Grand et al., 1979). Calmodulin, unlike Tn-C, does not bind to Tn-I in the absence of Ca^{++} (Amphlett et al., 1976).

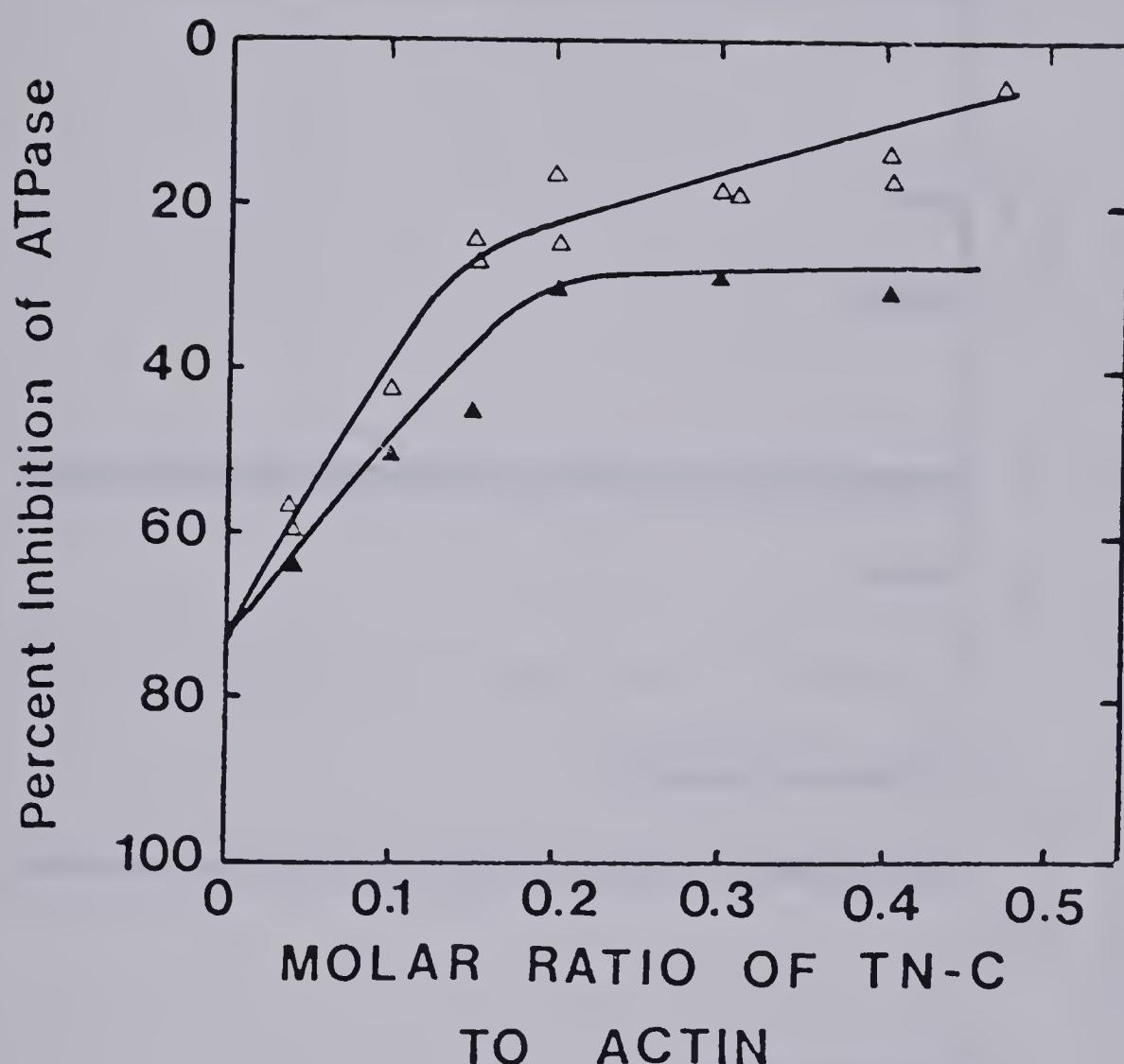


Fig. 60. Effects of increasing amounts of Tn-C on the actin activated ATPase of S-1 in the presence of platelet Tm and Tn-I, with (Δ) or without (\blacktriangle) Ca^{++} . The assay contained 0.12 mg/ml actin and 0.49 mg/ml S-1 in 2 ml of the standard buffer. Tn-I was at a 0.28 and platelet Tm at a 0.36 molar ratio to actin.

b)

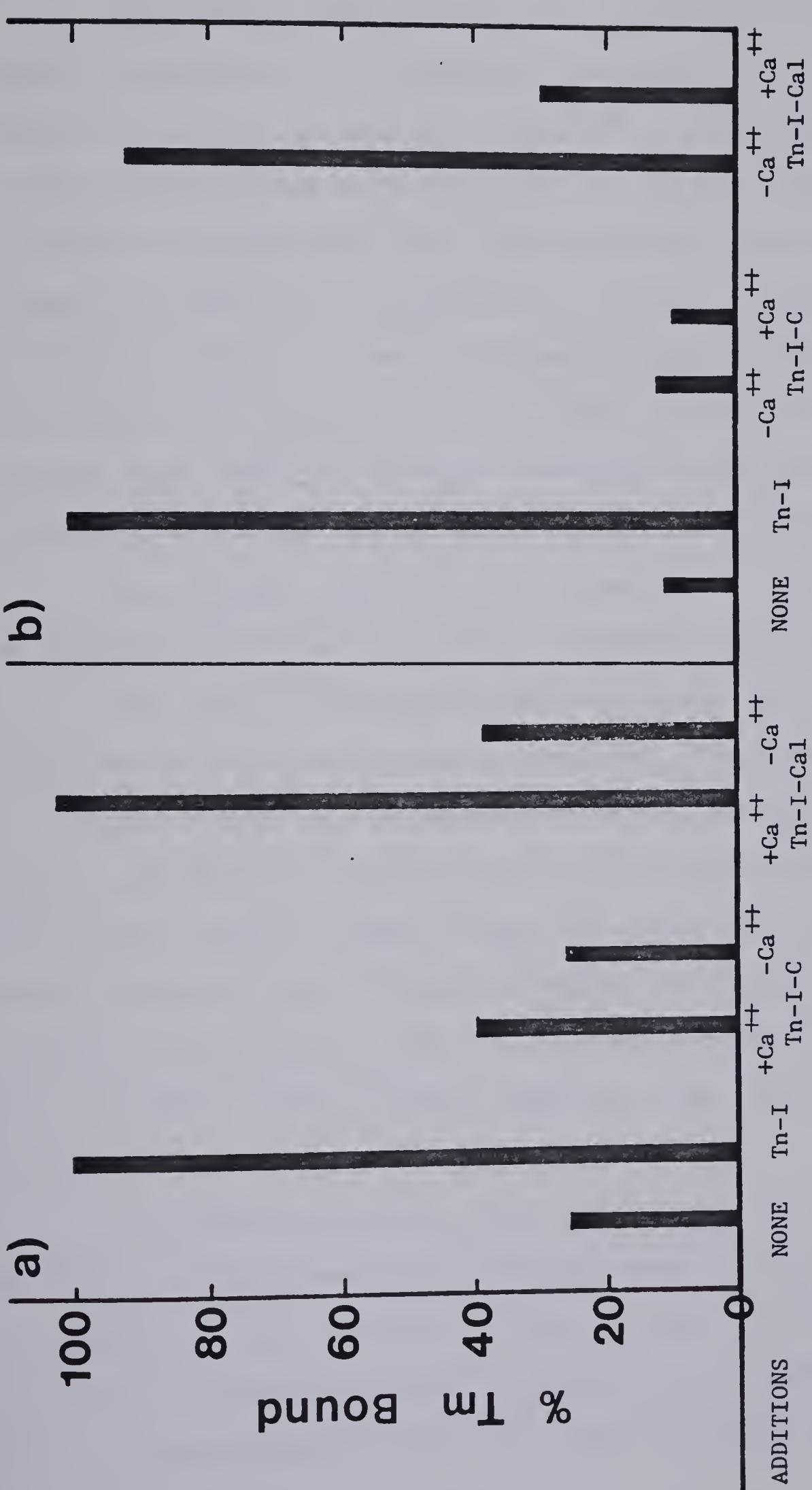


Fig. 59. The effects of Tn-I, Tn-C and calmodulin on the binding of (a) ^{125}I -platelet Tm or (b) ^{125}I -skeletal α Tm to F-actin. Values are expressed in terms of the radioactivity bound in the presence of Tn-I, which has been taken as 100%. In (a) each assay contained 0.2 mg actin, and a 0.2 molar ratio of platelet Tm to actin. Where added Tn-I = 0.3, Tn-C = 0.4 and calmodulin = 0.6 molar ratio to actin. In (b) actin was 0.2 mg and skeletal Tm = 0.2, Tn-I = 0.28, Tn-C = 0.5, and calmodulin = 0.6 molar ratio to actin. The buffer was 30 mM KCl, 2 mM DTT, 0.1 mM EGTA, 10 mM Tris, pH 7.5, and in (a) 2 mM MgCl_2 and in (b) no MgCl_2 .

ATPase assays demonstrated that in the absence of Ca^{++} calmodulin has no effect on the inhibition produced by Tn-I with either skeletal or platelet Tm (Fig. 61). When Ca^{++} is added, calmodulin, in much the same manner as Tn-C, is able to completely neutralize the inhibition observed with Tn-I and platelet Tm. Tn-C (Fig. 60) appears to be about twice as effective as calmodulin (Fig. 61) at neutralization, probably because of its stronger interaction with Tn-I. With skeletal Tm, calmodulin can only release the inhibition in the presence of Ca^{++} back to the level of inhibition observed with Tm alone (Fig. 61); a very similar effect as seen with Tn-C (Fig. 58).

These results show that a high degree of Ca^{++} sensitivity can be obtained with platelet Tm, Tn-I, and calmodulin without need of Tn-T. How is this Ca^{++} dependent response produced, and why is Tn-C unable to act in the same manner as calmodulin? The answer appears to be in the strength of the interactions of Tn-C and calmodulin with Tn-I. Generally, as the interaction between Tn-I and either of these Ca^{++} binding proteins becomes stronger, the result is that the interaction between Tn-I and the actin-Tm complex (and thus the inhibitory activity of Tn-I) becomes weaker. This is the basis of the proposed two site model of troponin action (Hitchcock et al., 1973; Potter & Gergely, 1974).

Tn-C interacts strongly enough with Tn-I even in the absence of Ca^{++} to not only lower Tn-I's inhibitory ability, but also to practically abolish its ability to induce platelet Tm to bind to actin (Fig. 59). Therefore, little Ca^{++} sensitivity can be obtained with Tn-I-C. Calmodulin on the other hand, does not interact with Tn-I in

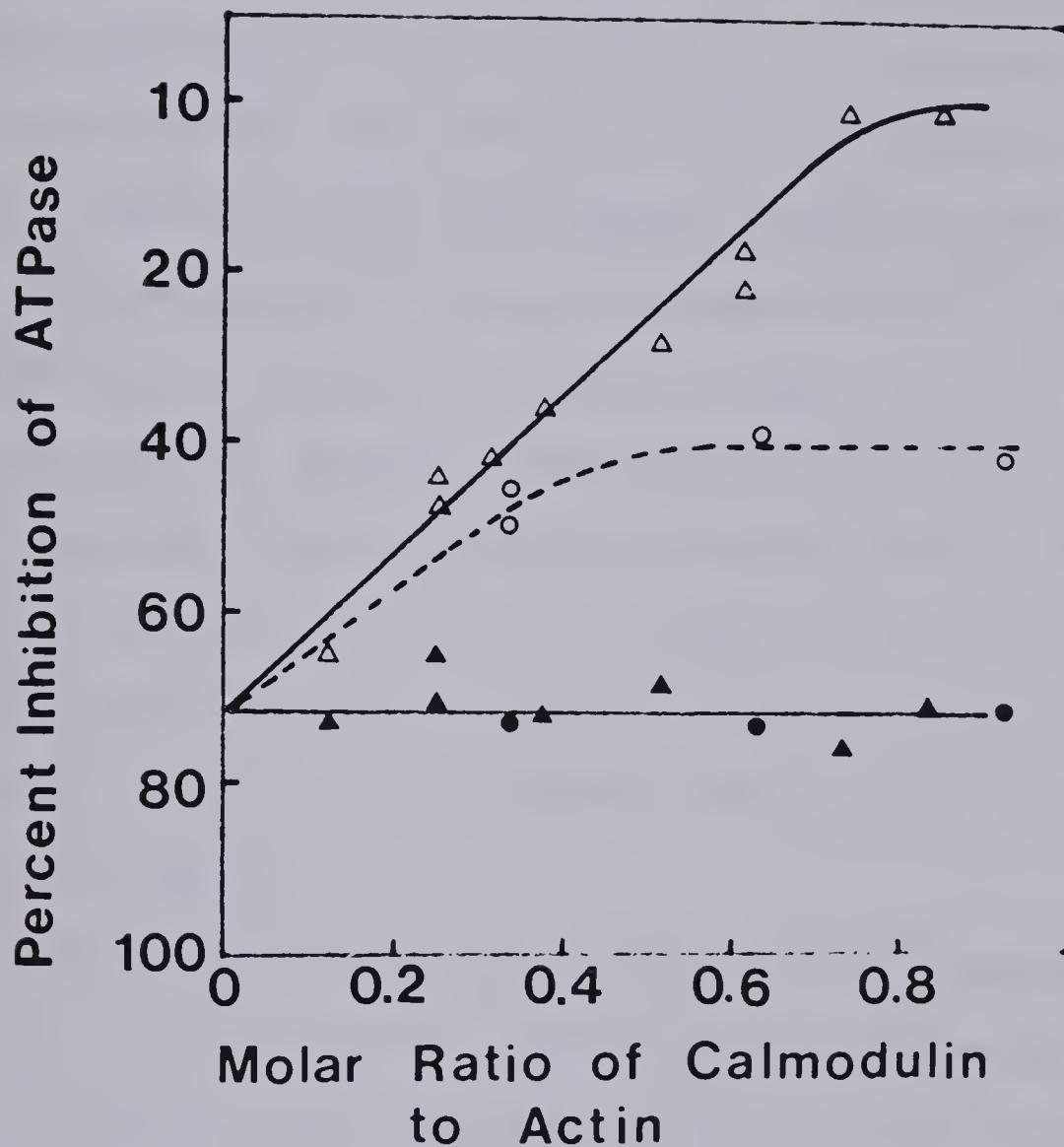


Fig. 61. Effects of increasing amounts of calmodulin on the actin activated ATPase of S-1 in the presence of either skeletal α (\circ , \bullet) or platelet (Δ , \blacktriangle) Tm with a 0.27 molar ratio of Tn-I to actin. Assays marked (\circ , Δ) contained Ca^{++} , while those marked (\bullet , \blacktriangle) did not. The skeletal Tm was at a 0.26 while the platelet Tm was at a 0.36 molar ratio to actin. Assays with skeletal Tm contained 0.25 mg/ml actin and 0.25 mg/ml S-1, while those with platelet Tm contained 0.12 mg/ml actin and 0.48 mg/ml S-1.

the absence of Ca^{++} , so that Tn-I remains free to hold platelet Tm onto the actin filament (Fig. 59), and to produce inhibition of the S-1 ATPase. Upon addition of Ca^{++} , calmodulin binds tightly to Tn-I, resulting in the detachment of platelet Tm from the F-actin filament (Fig. 59) and release of inhibition (Fig. 61). Presumably, the Tn-I-Cal complex also detaches from F-actin in a manner analogous to Tn-I-C plus Ca^{++} (Hitchcock et al., 1973; Potter & Gergely, 1974).

This mechanism of producing a response to Ca^{++} is entirely different than the mechanism proposed to account for the Ca^{++} sensitive regulation by troponin. The Tn-I-Cal system operates on an on-off principle, where the on position (bound to actin) produces inhibition, and the off position corresponds to free F-actin. The troponin system always remains bound to actin, and it is a shift in the position of Tm on the thin filament which produces either inhibition or activation.

Skeletal Tm did not give rise to a good Ca^{++} sensitive response with either Tn-I-C or Tn-I-Cal. This is because the assays were performed in a buffer which enabled skeletal Tm to bind to actin, which meant that the inhibition could never be released above the level of inhibition which skeletal Tm alone exhibits (40 to 60%). There is no reason, however, why Ca^{++} sensitivity could not be obtained with skeletal Tm and Tn-I-Cal if conditions are chosen so that the binding of skeletal Tm to actin is totally dependent on Tn-I. Fig. 59 demonstrates that Ca^{++} sensitive binding of skeletal Tm to F-actin can occur in the presence of Tn-I-Cal when there is no Mg^{++} present.

ATPase assays have been performed under these conditions

TABLE XIX

Correlation Between Ca^{++} Sensitivity and Binding
to Actin for Skeletal Tm

Conditions	Binding to Actin		Ca^{++} Sensitivity	Comments
	Without Ca^{++}	With Ca^{++}		
No free Mg^{++}	No	No	No	No inhibition with or without calcium
3 mM free Mg^{++}	Yes	Yes	No	Inhibition (40 to 60%) in both cases
No Mg^{++} or 3 mM Mg^{++} plus Tn-I	Yes	Yes	No	Inhibition (80 to 90%) in both cases
No Mg^{++} plus Tn-I-C	Poorly	No	Partial	Large amounts of Tn-I-C can produce some binding and inhibition
No Mg^{++} plus Tn-I-Cal	Yes	No	Yes	Good Ca^{++} sensitivity obtained (Amphlett et al., 1976)
3 mM Mg^{++} plus either Tn-I-C or Tn-I-Cal	Yes	Yes	Partial	Inhibition cannot be released above the level of Tm alone, which is still 40 to 60 % inhibited.
3 mM Mg^{++} plus troponin	Yes	Yes	Yes	Although remaining bound to actin Tm shifts its position to release inhibition.

(Amphlett et al., 1976), and the results are as expected. Very little Ca^{++} sensitivity is obtained with Tn-I-C, as no inhibition occurs even in the absence of Ca^{++} , while good Ca^{++} sensitivity is obtained with Tn-I-Cal.

A summary of the results obtained with Tn-I-C and Tn-I-Cal with skeletal Tm is presented in Table XIX. Platelet Tm under physiological conditions is most likely equivalent to the case for skeletal Tm in the absence of free Mg^{++} .

E. DISCUSSION

In this chapter the effects of platelet Tm on the ATPase activity of a skeletal muscle actin-myosin S-1 system were investigated. Many of the results which were obtained can be explained in terms of the binding of platelet Tm to actin, for Tm is only able to alter the Mg^{++} ATPase of S-1 indirectly, by affecting the attachment site for S-1 on actin.

Three different sets of ionic conditions were used for the experiments in this chapter. A situation corresponding to physiological conditions (150 mM KCl, 1 mM free Mg^{++}) was considered to be represented by 30 mM KCl and 3 mM free Mg^{++} , since these ion concentrations allowed skeletal Tm to bind to actin but not platelet Tm. Actual physiological ionic strength could not be used because of the inhibitory effect of KCl (or any salt) on the binding of myosin to actin. Buffers containing 8 mM free Mg^{++} were used when it was desired to induce the binding of platelet Tm to actin, while buffers containing 0.2 mM free Mg^{++} were used in order to prevent skeletal Tm from binding to actin. Neither of these last two buffers represents a physiological situation.

Skeletal Tm can bind to F-actin in two possible positions, one, inhibitory, outside of the actin groove, and the other, an active position, 1.5 nm closer to the center of the groove. When Ca^{++} binds to troponin, Tm shifts from the relaxed to the active location (Haselgrove, 1973; Huxley, 1973; Wakabayashi et al., 1975).

There are two means by which a change in the position of Tm may control muscle contraction. When bound in the inhibitory position skeletal Tm may physically block the attachment of myosin heads to actin, the block being released as Tm moves towards the actin groove. Alternatively, the movement of Tm may alter the organization or conformation of the F-actin monomers, changing their ability to activate myosin (Eaton, 1976; Yang et al., 1977).

When skeletal Tm and platelet Tm bind to actin, they both seem to prefer to bind at, or close to, the relaxed position. Skeletal Tm by itself inhibits the Mg^{++} ATPase of S-1 by up to 60%, while platelet Tm can inhibit the ATPase by 40% (Fig. 50). Skeletal Tm may act more effectively as a result of its head to tail binding, which may enable it to organize itself on the actin filament in a more ordered manner.

At low ATP concentrations both skeletal and platelet Tm are able to potentiate the ability of actin to activate the S-1 ATPase (Fig. 51). No direct evidence to indicate exactly what the potentiated state is has yet been obtained, but it would appear to depend on conformational changes induced by the binding of myosin heads to actin-Tm (Bremel et al., 1973). Experiments with platelet Tm in the presence of an enzyme system capable of maintaining a constant conc-

entrance of ATP (Bremel et al., 1973; Shigekawa & Tonomura, 1973) should be performed, and may provide further insight into the mechanism responsible for potentiation.

Generally, it is not possible to predict whether Tm, when added to F-actin, will activate or inhibit the S-1 ATPase. The observed effect appears to depend in a complex manner on the nature of the Tm, the actin and the myosin. For example, if skeletal actin is replaced by Acanthamoeba actin (Yang et al., 1977), or if skeletal myosin is replaced by smooth muscle myosin (Chacko et al., 1977), then skeletal Tm has the affect of activating the myosin ATPase, even at high concentrations of ATP. If gizzard Tm is substituted in place of skeletal Tm, then activation (2 to 3 times) rather than inhibition will take place with skeletal actin and myosin (Sobieszek & Small, 1977). Therefore, until experiments are performed with platelet actin and myosin it is not possible to state what effect platelet Tm by itself would have in vivo, if bound to actin filaments.

Upon addition of Tn-I to the assay the situation becomes much simpler. Tn-I seems to stabilize the binding of both platelet and skeletal Tm to the relaxed position on actin. Tn-I greatly increases the inhibitory power of platelet Tm (Fig. 56b), and can induce the protein to bind to actin in 3 mM Mg⁺⁺. The effects of Tn-I are completely analogous to its effects with skeletal Tm (Greaser & Gergely, 1971; Wilkinson et al., 1972; Shigekawa & Tonomura, 1973; Eisenberg & Kielly, 1974; Eaton et al., 1975). The ATPase studies support the results of the binding experiments with Tn-I (Chapter V), and suggest very strongly that platelet and skeletal Tm have a

common site of interaction with Tn-I.

The inhibition observed with Tn-I and either of the Tms is, of course, Ca^{++} insensitive. Under physiological conditions skeletal Tm requires the presence of the whole troponin complex in order to produce Ca^{++} sensitive regulation (Fig. 53 & 54). Tn-C (or calmodulin) in the presence of Ca^{++} can dissociate Tn-I from the thin filament, but skeletal Tm still remains bound close to the inhibitory position, and inhibition is observed (Fig. 58 & 61). The presence of Tn-T provides a connection between troponin and skeletal Tm, which is required in order to transmit the conformational change induced in Tn-C by binding Ca^{++} to skeletal Tm, forcing the Tm out of the relaxed position into the higher energy activated position.

Perhaps because this connection is missing good Ca^{++} dependent regulation cannot be obtained with platelet Tm and skeletal troponin (Fig. 53 & 54). The conformational change undergone by Tn-C cannot be used to alter the position of platelet Tm on the thin filament, but probably does result in the partial release of inhibition which is always observed when Ca^{++} is added (Fig. 55).

From the results of the ATPase assays (and from preliminary binding studies) it appears that troponin can hold platelet Tm onto the actin filament in the presence and absence of Ca^{++} . This is surprising because Tn-T does not interact with platelet Tm, and the other two troponin components (Tn-I-C) cannot, by themselves, effectively induce platelet Tm to bind to actin (Fig. 59). This means that Tn-T must either bind to actin (for which there is some evidence, Potter & Gergely, 1974; Hitchcock, 1975), or prevent Tn-C

from neutralizing Tn-I's ability to bind to actin-Tm, a role Tn-T plays in skeletal muscle, at least in the absence of Ca^{++} (Eisenberg & Kielly, 1974). Tn-T may perhaps perform the same function with platelet Tm even in the presence of Ca^{++} , possibly because platelet Tm and Tn-T do not change their position on the actin filament.

In disagreement with the results reported here, Fine et al. (1973) have provided evidence to indicate that a non-muscle Tm isolated from chicken brain can substitute for skeletal Tm in the regulation of a skeletal actomyosin system (results shown in Table XX). As with platelet Tm the brain Tm is able to increase the inhibition observed with troponin, but, unlike with platelet Tm, the inhibition with brain Tm appears to be almost fully released in the presence of Ca^{++} .

It is of course possible that the use by Fine et al. (1973) of chicken brain Tm rather than horse platelet Tm, or their use of chicken skeletal troponin in place of rabbit skeletal troponin (the two proteins have different M.Wt. subunits) could account for the difference in results obtained. However, criticisms which can be made of the ATPase assays of Fine et al. (1973) may cast some doubts upon the meaning of their results.

Firstly, it is clear that Fine et al. (1973) have not performed as extensive a series of assays as have been reported here. They have not attempted to vary the amounts of Tm and troponin added to the assay to determine what effect this has upon their results, but in fact have chosen molar ratios of the regulatory proteins which are quite unphysiological. There is 1.0 molecule of the

troponin complex for every 1.6 F-actin monomers, but only about 1.0 Tm molecule present for every 10 F-actin monomers; that is, there is a great excess of troponin, yet not enough Tm to even cover all available actin filaments! Under these conditions the behaviour of the troponin component of the regulatory system may be expected to predominate, and it may be difficult to measure the effects of the Tm.

The major difference between the results with brain and platelet Tm is the extent to which inhibition is released in the presence of Ca^{++} . Fine et al. (1973) have not indicated the rate obtained with actin and myosin alone; the rate which must be considered as the uninhibited ATPase activity of the system (Table XX). With 4 mM free Mg^{++} in the assays, the activity shown with actin and myosin plus skeletal Tm probably constitutes an inhibited rate, but by how much is not clear. Therefore, it is difficult to determine how well brain Tm is in fact releasing the inhibition in the presence of Ca^{++} . In this assay (Table XX) the inhibition is due mainly to the troponin complex, and it is not obvious how brain Tm could completely release this inhibition (even if it could) when there is not enough of it present to possibly interact with all the F-actin monomers inhibited by troponin. Certainly the assays with brain Tm should be repeated in more detail.

In Fig. 53 and 54 it can be seen that certain ratios of skeletal Tm to troponin are able to activate the Mg^{++} ATPase of S-1 well above the level observed for actin and S-1. This dependency of the activating effect of Tm and troponin on the molar ratio of

TABLE XX

Effect of Chicken Muscle Tm and Chicken Brain Tm on the ATPase Activity of an Actomyosin System in the Presence of Chicken Muscle Troponin [from Fine et al., 1973]

Components Added ^b	ATPase Activity ^a	
	Without Ca ⁺⁺	With Ca ⁺⁺
Muscle Tm	0.37	0.37
Muscle Tm + Troponin	0.10	0.44
Troponin	0.20	0.22
Brain Tm + Troponin	0.15 0.14	0.36 0.36

^a Activity in μ moles PO₄/minute/mg S-1

^b The assay buffer was 40 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 0.7 mM ATP; for + Ca⁺⁺ 0.2 mM CaCl₂ was added.

The assay contained 0.7 mg rabbit muscle myosin, 0.28 mg rabbit muscle actin, 0.046 mg Tm and 0.25 mg troponin. Activity measured by the pH-stat method.

skeletal Tm to F-actin and troponin has not previously been described in the literature, but one possible explanation can be given in terms of the potentiating abilities of troponin and Tm.

Troponin by itself has no potentiating ability, so at the start of the curves, in the absence of Tm, no activation above 100% is observed. However, troponin and Tm together are able to enhance the S-1 ATPase activating ability of F-actin, and in fact troponin acts to extend the potentiating effect of skeletal Tm to higher ATP concentrations, perhaps 1 mM or more (Shigekawa & Tonomura, 1973; Bremel et al., 1973). The activation of the Mg^{++} ATPase of S-1 increases then until all actin filaments containing troponin are regulated in a cooperative manner by skeletal Tm, but after this point, what causes the potentiation to once again decrease?

A clue may be given by the fact that the activation at a 1:7 molar ratio of troponin to actin drops much faster than the potentiation at a 3:7 molar ratio.

In the absence of Ca^{++} troponin is thought to be bound to the thin filament through two sites, Tn-I and Tn-T, but in the presence of Ca^{++} it is thought that the Tn-I site is lost (Hitchcock et al., 1973; Potter and Gergely, 1974; Hitchcock, 1975). Therefore, if troponin is only bound to the thin filament through Tm in the presence of Ca^{++} , it seems reasonable to assume that excess Tm free in solution should be able to compete effectively with the actin bound Tm for the troponin. If through such competition troponin is removed from the actin filament, the drop in activation as excess skeletal Tm is added can be explained, for as troponin is removed, the

ATP concentration at which potentiation occurs drops from about 1 mM back to the level needed for activation with Tm alone (about 0.1 mM) a level which would not be reached in this type of assay.

At a high molar ratio of troponin to actin (Fig. 53) more skeletal Tm would have to be added than at a low molar ratio (Fig. 54) before troponin would start to be removed from the F-actin.

Experiments to support this explanation should be performed, perhaps with ¹²⁵I-labelled troponin, to determine if free skeletal Tm can in fact remove troponin from the thin filament in the presence, but not in the absence, of Ca⁺⁺. Such experiments could also provide further support for the two site model of troponin action (Hitchcock et al., 1973; Potter & Gergely, 1974).

Platelet Tm could not provide Ca⁺⁺ sensitivity with either skeletal troponin or Tn-I-C (Fig. 60), but could with a mixture of Tn-I and calmodulin (Fig. 61). A schematic diagram of the proposed mechanism is shown in Fig. 62. This on-off model would not seem to be of relevance to the situation involving skeletal Tm in vivo because skeletal Tm, alone, binds to actin under physiological conditions. It is possible, however, that the on-off mechanism has a place in the regulation of contraction in non-muscle cells.

Platelet Tm does not bind to F-actin under physiological conditions, and so may need to be held on by another protein. If this protein was capable of binding platelet Tm to the inhibitory position on the actin filament, and of binding to calmodulin in the presence of Ca⁺⁺, a simple regulatory system would be formed. A protein homologous to Tn-I is the logical candidate for this role, especially

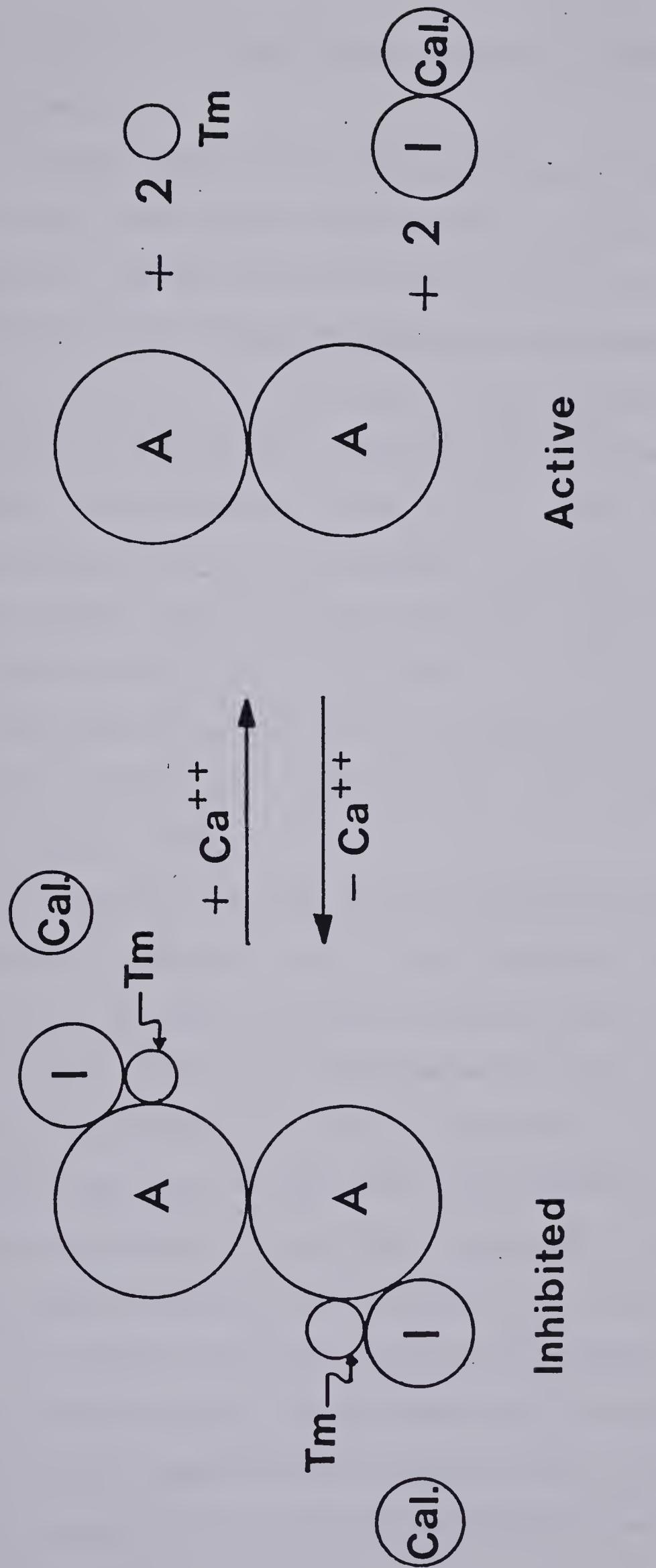


Fig. 62. A possible on-off mechanism by which a non-muscle Tm (Tm), an inhibitory protein (I) and calmodulin (Cal) may confer Ca^{++} sensitivity to a non-muscle actin-myosin system. The on position corresponds to the inhibited state, while the off position is equivalent to free F-actin (A). filaments.

as the ability to interact with Tn-I has been so highly conserved in platelet Tm.

However, even if a Tn-I like protein is not found in non-muscle cells there are many other non-muscle proteins capable of forming Ca^{++} dependent complexes with calmodulin, few of which have been purified, and none of which have yet been tested for their ability to act in an actomyosin ATPase system (Sharma et al., 1978, 1979; Grand & Perry, 1979). Calmodulin itself may not be an essential component of the regulatory system, since it appears that some actin binding proteins (of which actinogelin is the only one so far isolated (Mimura & Asano, 1979)) are able to form Ca^{++} sensitive complexes with actin in the absence of calmodulin. It is interesting that Ca^{++} always acts to release the actin binding protein from the actin (Condeelis & Taylor, 1977; Brotschi et al., 1978; Bryan & Kane, 1978; Mimura & Asano, 1979).

In summary, if Tm does act in non-muscle cells as a member of a contractile regulatory system, it may perform its role by alternately associating and dissociating from the actin filament, probably through Ca^{++} dependent interactions with other actin binding proteins. Such a mode of action provides a rational explanation for the two most important differences between platelet and skeletal Tm. To function in this manner platelet Tm must bind very weakly to actin, so it is smaller than the muscle Tm and has lost at least one actin binding site. To regulate contraction by the on-off mechanism platelet Tm does not need to move over the surface of the F-actin filament, and so it is not necessary that it bind to Tn-T; thus the Tn-T binding site has been deleted from the platelet Tm molecule.

The on-off mechanism of regulation by non-muscle Tms would be well in keeping with the picture of labile, constantly reorganizing actin filaments that we have for non-muscle cells. A tropomyosin-skeletal Tm regulatory system, permanently bound to the actin filament, may have a place only in muscle cells, where the actin filaments themselves are fixed in unchanging positions.

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